STRUCTURAL STUDIES ON THE IRON STORAGE PROTEIN, APOFERRITIN

by

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ABSTRACT

Structural Studies on the Iron-Storage Protein, Apoferritin

Margaret Paterson

Horse spleen apoferritin consists of 24-subunits symmetrically arranged around a central iron-containing cavity. It was originally thought that the 24 subunits were identical, however evidence based on SDS-PAGE and isoelectrofocusing led Drysdale to propose a model with two distinct subunits, the L subunit (m.wt. 19,000) and H subunit (m.wt. 21,000) which are combined in different proportions to give various 24-mers (isoferritins). This model has been disputed by several workers.

When horse spleen apoferritin is electrophoresed by SDS-PAGE it resolves into several bands. The origin of these bands were studied. Bands were visible with molecular weights above and below that of the subunit. Using a 2-dimensional technique several of these band could be attributed to inter-molecular disulphide bonds forming dimers and trimers and an intra-molecular disulphide bond giving an apparently smaller subunit band (m.wt. 15,000). Other smaller molecular weight bands have been re-assessed by SDS-PAGE to have molecular weights of 11,000 (peptide B) and 4,500 (peptide C). Amino acid analysis indicates peptide C arises from the C-terminal end of the molecule. Sequencing of this peptide gave the following sequence:

leu-thr-leu-lys-aromatic-trp

which corresponds to the last six amino acids of the published sequence. Peptide C could therefore extend beyond this sequence and one explanation of this could be that the H subunit is a precursor of the L subunit.

A glycoprotein affinity column showed that glycosylated subunits did occur but the extent of glycosylation was insufficient to affect the subunit molecular weight on SDS-PAGE but may be sufficient to influence isoelectro-focusing profiles.

Solid-phase sequencing studies carried out on isolated L and H-rich subunits showed that both were resistent to sequencing from the N-terminal end. This implies that H as well as L subunits are H-blocked.

Chemical modification, cleavage and spectrophotometric studies indicated the presence of 1 tryptophan, 5-6 tyrosine and 2 cysteine residues which corresponds well with the published sequence.

Tissue culture experiments carried out on Morris hepatoma cells showed ferritin synthesis could be increased up to 5-fold greater than control cells by introducing iron

into the culture medium. Initial studies were also carried out to determine if either subunit (L or H) was preferentially synthesized during iron loading. Studies of this type may provide information on whether two subunits do exist and if so their function within the molecule.

INTRODUCTION

INTRODUCTION

Iron is an essential trace element required by nearly all living species. It is involved in electron transport chains, the transport of oxygen by haemoglobin and is a component of many enzymes. In all these instances iron is bound to the molecule by a variety of organic ligands and in these complexes it is able to form a specific, controlled biological function. Free iron, on the other hand, is toxic to the cell's metabolism as it can lead to the denaturation of proteins. Its presence in the cell may result in the formation of superoxide by transfer of a single electron to an oxygen molecule and this can result in the destruction of other cellular constituents such as lipid and nucleic acids (Aisen, 1977). The cell thus needs to maintain a supply of iron in order to synthesize the biologically important compounds of which iron is a component yet protect itself from the harmful effects of free iron.

In order to meet the metabolic demands of the organism, iron must be acquired from the environment and delivered to the sites where it is required. In the unicellular organisms this may be a relatively simple system but in higher organisms a more complex mechanism is needed. The metal has to be transported to cells which need it for the synthesis of iron-containing molecules and at the end of the life-span of these molecules the iron

must be reprocessed and conserved. Any excess must be removed either by excretion or by internal storage in the iron storage protein, ferritin. Up to 70% of the total body iron is present in haemoglobin and the major recycling of iron in the body involves the production, circulation and degradation of this molecule. As synthesis of haemoglobin occurs in the bone marrow and reticuloendothelial cells breakdown the molecule in the liver and spleen, iron must be transported from the spleen and liver back to the bone marrow for the cycle to continue. This is achieved by the iron transport protein, transferrin. Serum transferrin is a glycoprotein which is synthesized in the liver and its single polypeptide chain contains two binding sites for Fe(III). In man, approximately 0.9 mg of iron enter the body daily through the walls of the intestine but the daily exchange of iron may be as much as 37.5 mg (Cook, Finch and Smith, 1976). Iron is continually being transported by transferrin from the sites of ingestion to cells where it may be required for synthesis, breakdown, storage or excretion as shown in Figure 1.

The total iron content of the human body is estimated to be about 3 - 4 g. As mentioned previously a large proportion, 68%, is present in haemoglobin whilst about 10% is found in specific tissues such as muscle in the form of the protein myoglobin. A small percentage is also contained in the cytochromes and in enzymes such as

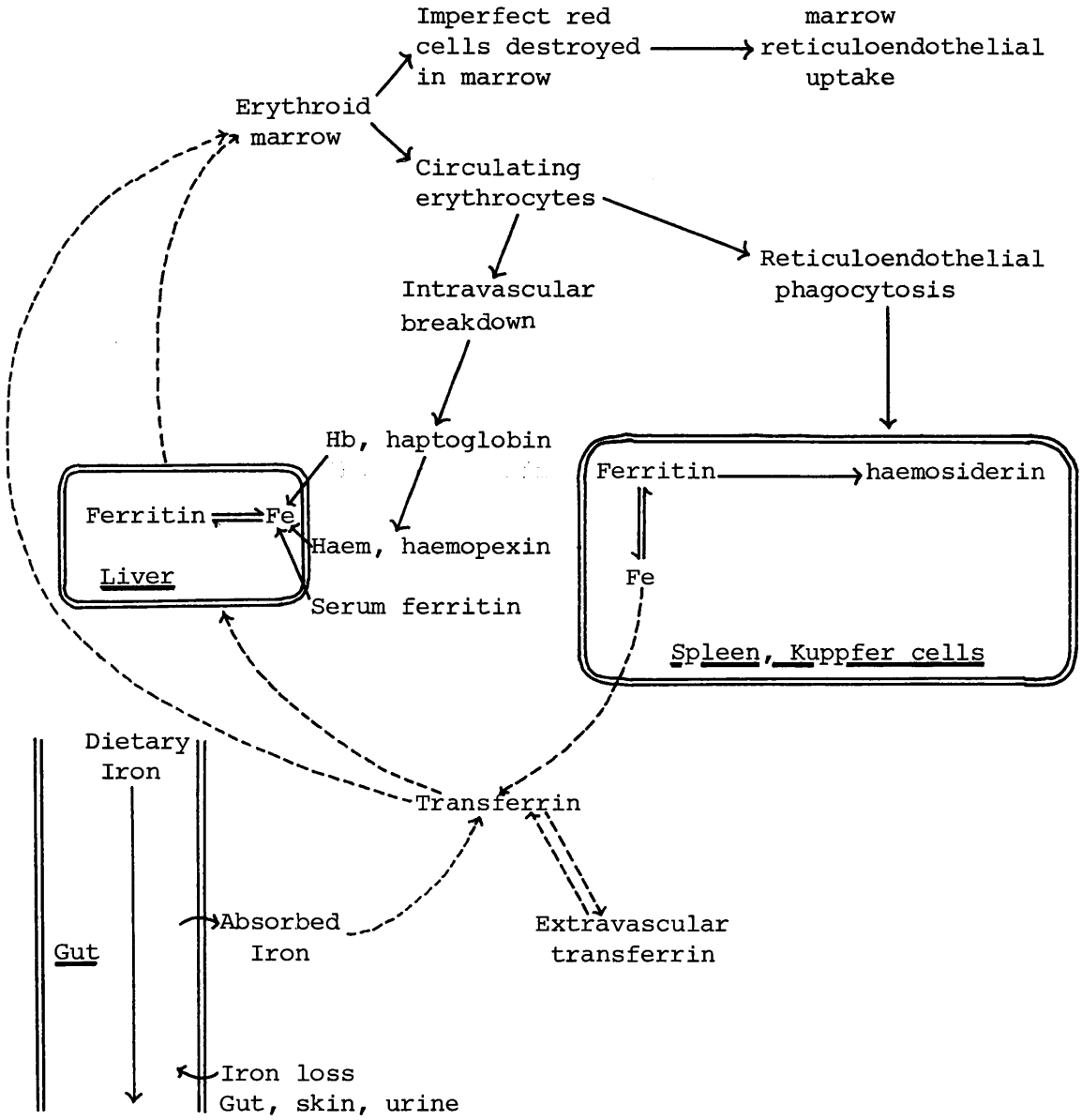


Figure 1: Iron exchange within the body. The dotted lines show iron transported by transferrin

From: Munro and Linder, 1978.

catalase. The storage forms of ferritin and haemosiderin account for the remaining 12%.

Because of its ability to change its valency state with comparative ease, iron is exploited by many enzymatic systems to catalyze oxido-reductase mechanisms. The main classes of iron-containing proteins are listed in Table 1. Such proteins are intimately associated with both the physical and chemical activities that constitute the normal functioning of the cell.

While some proteins serve as important structural elements of the body (hair, collagen), others may be enzymes, hormones, proteins associated with genes (histones) oxygen carriers, proteins that are concerned with immunological defence (antibodies) and a variety of other functions. It has become the object of the protein chemist to attempt to explain the particular physiological and biochemical functions of these macromolecules in terms of their molecular structure. The theme of the present project was to investigate various structural aspects of the iron-storage protein in the light of such a structure-function relationship.

Ferritin, which is the major iron storage protein was first described as a discrete entity by Laufberger in 1937 although the deposition of iron in tissues had long been recognized under the name of haemosiderin (Laufberger, 1937). The ferritin molecule consists of a

Protein	Haem(H) or Non-Haem(N)	Function
Haemoglobin Myoglobin	H	O_2 carrier
Hydroperoxidases (catalase, peroxidase)	H	Enzymes with H_2O_2 as substrate
Cytochromes	H	Electron carriers
Cytochrome C oxidase	H	Terminal oxidase
Flavoprotein dehydrogenases and oxidases (1)	N	Oxidising enzymes
Oxygenases(2)	N(3)	Enzymes catalysing incorporation of O_2 into substrates
Ferredoxin	N	Electron carrier
Haemerythrin	N	O_2 carrier
Transferrins (blood, egg white milk)	N	Transport, storage
Ferritin	N	Transport, storage

(1) This enzyme contains copper as well.

(2) Not all members of this class are iron-proteins.

(3) One oxygenase (tryptophan 2,3-dioxygenase) is a haem-protein.

Table 1: The main clases of iron-containing proteins

From:Malmstrom, 1969.

multisubunit protein shell surrounding a central core of hydrous ferric oxide-phosphate as shown schematically in Figure 2.

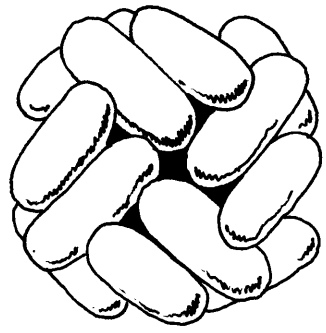


Figure 2: Molecule of horse spleen ferritin. The molecule is a symmetrical shell of 24 protein subunits.

The protein shell protects the cell from any harmful effects of free iron and six channels within the shell are thought to allow iron to enter and leave the molecule as required by the cell's metabolism. Whereas ferritin is a multisubunit protein which has a well defined appearance under high resolution electron microscopy, haemosiderin is characterized by its heterogeneity in composition. It probably represents a degraded form of ferritin arising as a result of lysosomal digestion of parts of the protein shell. This relationship between ferritin and haemosiderin is shown in Figure 3.

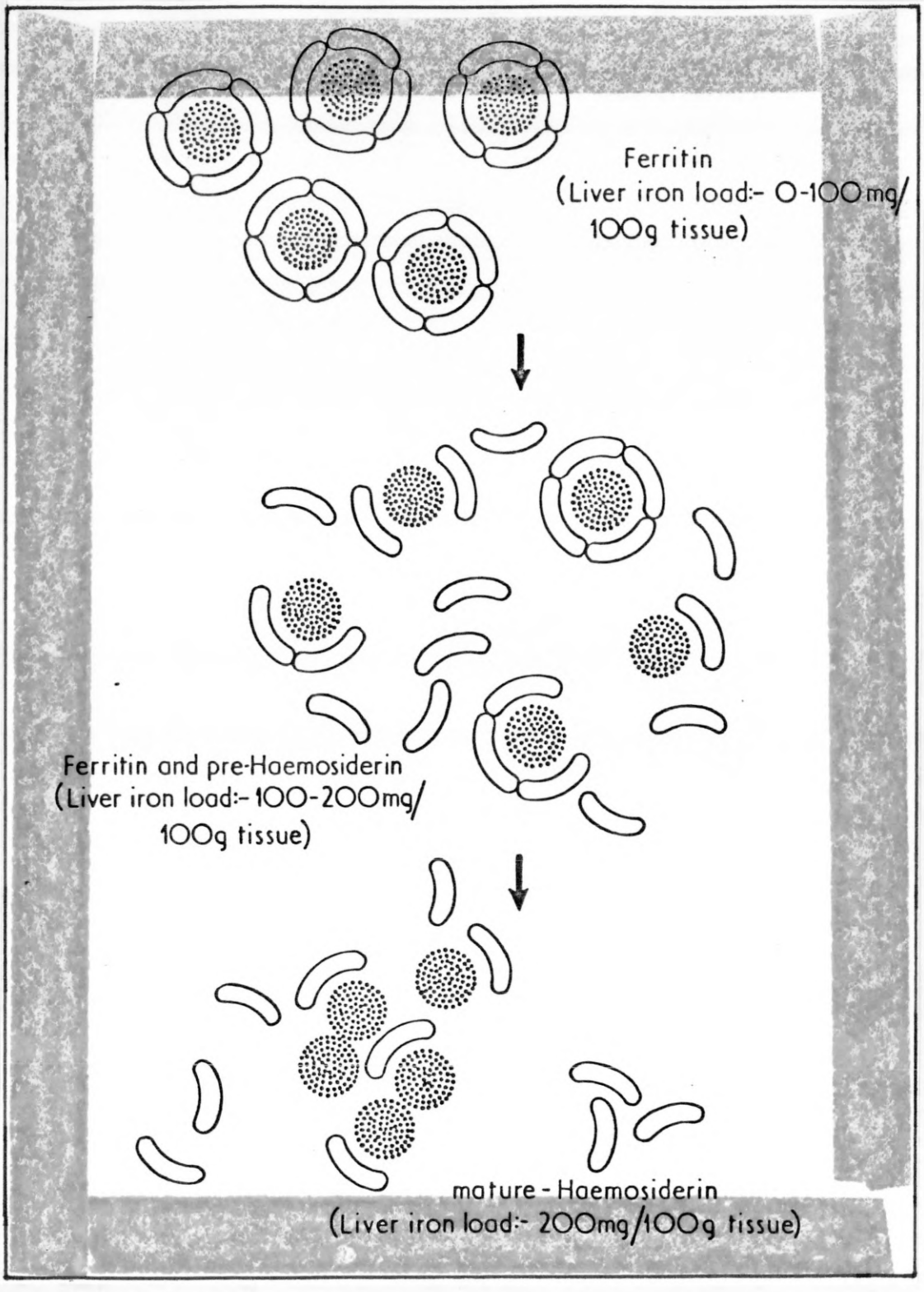


Figure 3: The relationship between haemosiderin and ferritin.

The ferritin molecule appears to have developed fairly early in evolution and is found in many organisms including fungi (Bozarth and Goenaga, 1972; David and Easterbrook, 1971), annelid worms (Linder, 1963), fish (Kato and Shimada, 1970) and mammals. Phytoferritins have been reported in various plants and legumes (Crichton *et al*, 1978; Seckbach, 1968). Recently ferritin-like molecules have been found in two species of bacteria namely *Escherichia coli* (Bauminger *et al*, 1980) and *Azotobacter vinelandii* (Stiefel and Watt, 1979). In mammals, ferritin is predominately found in the spleen and liver but is also present in a wide variety of other tissues including bone marrow, kidney, and heart (Alfrey, Lynch and Whitley, 1967; Munro *et al*, 1975) but it can be detected in most cells including tumour cells (Richter, 1965). Using sensitive radioimmunoassay procedures, ferritin has now been detected in serum and the levels of ferritin circulating in serum can be used as an indication of body iron stores (Addison *et al*, 1972; Jacobs, 1977).

If a sample of ferritin is fractionated on a molecular weight basis a distribution profile similar to that in Figure 4 may be obtained. Such a profile is indicative of a heterogeneous species. Rothen (1944) was able to demonstrate that the slower moving sharp peak was in fact a homogeneous protein. This colourless fraction contained the iron-free protein called apoferritin. This is the

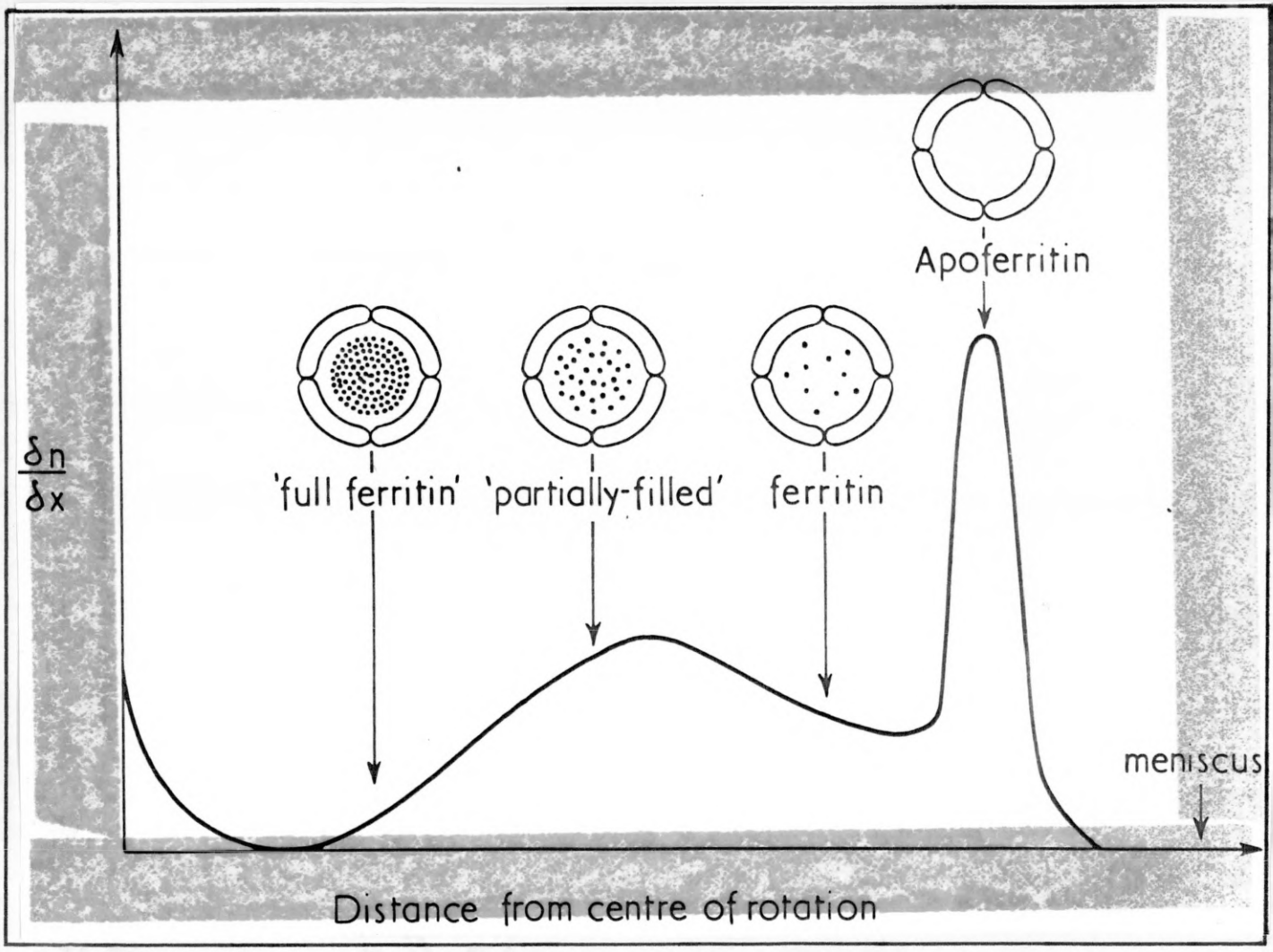


Figure 4: A typical distribution profile obtained by sedimenting a sample of horse spleen ferritin.

hollow protein shell devoid of any iron. Thus the heterogeneity exhibited by ferritin was assumed to result from varying amounts of iron contained within the protein shell. A sample of ferritin would, therefore, contain molecules which differed in their iron content but not in the composition of their protein shell.

Isolation and Purification of Ferritin

The isolation of ferritin is a relatively simple procedure when compared to many other protein and enzyme purifications. Ferritin is a large, water-soluble molecule which is stable at temperatures of 70°C and these features are exploited in its preparation. A detailed description of the method is given in Figure 17 of the Materials and Methods section.

Normally the tissue is homogenised in approximately 5 volumes of water or dilute buffer solution and the cell debris removed by centrifugation. The protease inhibitor phenyl methyl sulphonyl fluoride is now often added at this stage to prevent any proteolysis. The next stage in the isolation procedure takes advantage of the proteins stability to heat as the supernatant is rapidly heated to 70°C for 10 minutes then cooled on ice. The coagulated protein is removed by centrifugation and the ferritin which is present in the supernatant is precipitated by ammonium sulphate. After centrifugation, ferritin is isolated from the pellet by a combination of methods including ultracentrifugation, column chromatography, preparative electrophoresis, and affinity chromatography but each of these methods has its drawbacks. Ultracentrifugation tends to select ferritins with a high iron content while preparative electrophoresis and affinity chromatography may select

certain isoferritins (see later) due to differences in the surface charge of the molecule.

Cadmium sulphate crystallization may also be used as a purification procedure as ferritin can be crystallized from a 4 - 5% (w/v) cadmium sulphate solution. If this method is used, the bound cadmium can be removed by treatment with neutral bisulphite (Hegenauer, Saltman and Hatlen, 1979).

Ferritin Structure

The ferritin molecule has a remarkably constant structural design throughout the biological world. There are several structural features which appear to be common to all ferritins with the mammalian ferritins resembling each other more closely than the phytoferritins found in the plant kingdom. Most of the structural studies on ferritin have been carried out on horse spleen ferritin.

Because of the heterogeneity displayed by ferritin, as can be seen in its sedimentation pattern in Figure 4, a calculation of its molecular weight is meaningless as it is dependent on the amount of iron contained within the protein shell. However the molecular weight of apoferritin has been determined by a number of workers to have a molecular weight in the range 430,000 to 465,000 as shown in Table 2.

The protein moiety of horse spleen apoferritin forms a nearly spherical hollow shell with an external diameter of 130 Å and a central cavity of 75 Å (Harrison, 1963). This cavity has eight shallow pockets (Treffry *et al*, 1977) which can contain up to 4500 atoms of iron in the form of ferric oxyhydroxide hydroxyphosphate $(FeOOH)_8(FeO{:}OPO_3H_2)$ although the average number of iron atoms is usually 2,500 thus leaving the molecule with a reserve capacity for iron uptake. Electron

	Molecular Weight	Method of Determination
Apoferritin:		
	430,000	Light scattering(1)
	462,000	X-ray diffraction(2)
	465,000	Sedimentation diffusion(3)
	465,000	Sedimentation equilibrium(4)
	443,000	Sedimentation equilibrium(5)
Subunit:		
	18,200	Gel-filtration of columns of Bio-gel A-5M in 6M guanidine hydrochloride (4,8)
	18,300	Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (6,7)
	18,400	Sedimentation equilibrium in glycine buffer after dissociation in 67% acetic acid (7)
	18,700	Sedimentation equilibrium 6M guanidine hydrochloride (7)
	18,800	Gel-filtration on columns of Sepharose 6B in 6M guanidine hydrochloride (7,8)

Table 2: Molecular Weight of Apoferritin and its Subunit.

(1) Fischbach and Anderegg, 1965

(2) Harrison, 1959

(3) Rothen, 1944

(4) Björk and Fish, 1971

(5) Crichton, Eason, Barclay and Bryce, 1973

(6) Crichton and Bryce, 1970

(7) Bryce and Crichton, 1971a

(8) Bryce and Crichton, 1971b

density maps have revealed the presence of six channels which lie along the molecular 4-fold axis (Banyard, Stammers and Harrison, 1978) and presumably these provide a route by which iron and other small molecules may enter and leave the central cavity. Although it was originally thought that the ferritin molecule consisted of 20 subunits each with a molecular weight of 23-25000 (Hofmann and Harrison, 1963), the emergence of new data based on gel filtration, sedimentation velocity and SDS-polyacrylamide gel electrophoresis led to a re-assessment of the subunit molecular weight. The values which were obtained for the molecular weight of the subunit using these various techniques are given in Table 2. The recalculated value of 18500-19000results in a 24 subunit model which corresponds to the lattice symmetry of close packed cubic crystals which both ferritin and apoferritin are able to form. The subunits are arranged in 432 symmetry and are equivalent in their orientation in space (Hoare, Harrison and Hoy, 1975). The basic model of apoferritin containing 24 subunits is now well established, however, the possibility of these subunits being chemically and structurally equivalent has recently been questioned. Several workers have obtained multiple bands using isoelectrofocusing (Adelman, Arosio, Drysdale, 1975; Powel *et al*, 1974) and electrophoresis (Adelman, Arosio and Drysdale, 1975; Ishitani, Niitsu and Listowsky, 1975) on a variety of ferritins and apoferritins from a number of different sources. The term isoferritin has been used

to define ferritins of different isoelectric points but with the same function, in analogy with the isoenzymes. In polyacrylamide gels containing sodium dodecyl sulphate (SDS), bands have been observed which have lower molecular weights than that of the subunit. These are thought to represent specific fragments of the subunit and have been called peptide B and C with molecular weights of 11,000 and 7,000 respectively. The fact that these bands are observed in apoferritins from a variety of organs and species suggests that they are formed by a specific proteolytic cleavage of the subunit. Incorporation of a serine protease inhibitor to all stages of the isolation procedure significantly reduces the appearance of these bands when analysed by SDS electrophoresis. This is verified by amino acid analysis of these fragments, the sum of which gives good correlation with the amino acid of the subunit as shown in Table 3 (Collet-Cassart and Crichton, 1975).

The amino acid composition of apoferritins from various sources are given in Table 4. As can be seen from this table, although the individual amino acid compositions of ferritins isolated from different sources do vary, their overall composition is remarkably similar. In general, there is a high percentage of non-polar amino acid residues whilst the three amino acids which strongly support α-helix formation namely leucine, glutamic acid (or glutamine) and alanine (Chou and Fasman,

Amino acid composition of peptides B and C

Amino acids	Peptide B	Peptide C	B+C	Apoferritin
ASX	11.8	6.0	17.8	17.3
THR	3.8	2.5	6.3	5.5
SER	6.6	5.1	11.7	9.0
GLX	17.6	7.2	24.8	23.9
PRO	1.5	2.1	3.6	2.8
GLY	7.0	4.2	11.2	9.9
ALA	10.2	5.0	15.2	14.0
VAL	4.6	3.6	8.2	6.9
MET	2.2	1.1	3.3	2.8
ILE	2.3	1.7	4.0	3.5
LEU	16.2	6.4	22.6	25.0
TYR	3.2	2.0	5.2	5.0
PHE	4.8	2.1	6.9	7.3
HIS	4.8	1.2	6.0	5.8
LYS	6.3	1.7	8.0	8.7
ARG	5.8	2.5	8.3	9.5

Table 3: Amino acid composition of a subunit of horse spleen apoferritin and the two smaller peptides B and C.

From: Collet-Cassart and Crichton, 1975.

	HuL	HuS	HuH	HoL	HoS	HoH	RaL	RaS	RaM	Lentil	Pea	Phyc.
Molecular weight	18,000 -19,000	18,000 -19,000	18,300	18,000 -19,000	18,500	18,000 -19,000	19,000	19,300	19,200	20,300	21,400	18,000
CYS	1.5	1.7	ND	2.6	2.9	ND	ND	ND	ND	ND	ND	ND
ASX	19.2	19.3	19.0	17.9	17.3	18.4	19.6	20.4	21.2	30.3	20.9	17.1
THR	6.2	6.1	7.6	5.6	5.5	7.7	6.6	6.6	8.4	4.0	5.0	8.2
SER	9.3	7.7	7.8	8.9	9.0	11.2	9.1	9.0	11.8	14.7	9.7	11.0
GLX	23.9	22.3	24.5	25.3	23.9	19.9	25.1	26.2	24.2	28.9	27.6	25.9
PRO	2.9	3.8	5.7	3.1	2.8	6.4	4.7	4.2	5.5	3.1	6.0	3.5
GLY	10.1	10.8	11.0	10.2	9.9	10.2	10.5	11.9	11.5	11.0	9.6	12.5
ALA	13.7	13.8	15.3	13.4	14.0	12.7	14.3	14.1	13.6	14.7	15.4	15.1
VAL	6.3	6.0	7.2	6.9	6.9	7.4	6.9	7.2	7.9	12.8	17.4	8.3
MET	2.9	2.7	1.9	2.5	2.8	4.8	2.4	1.6	2.7	3.0	3.9	1.4
ILE	2.5	3.8	4.7	3.6	3.5	4.9	3.1	3.1	4.0	7.4	6.9	7.9
LEU	23.4	23.3	24.0	24.2	25.0	20.3	23.9	23.4	19.1	15.8	17.4	18.8
TYR	6.0	4.4	5.2	4.1	5.0	4.8	4.0	3.1	5.1	6.0	6.5	2.7
PHE	6.9	7.0	6.0	7.7	7.3	7.3	6.7	6.6	5.8	8.4	9.5	3.1
TRP	2.2	2.2	ND	2.1	2.1	ND	ND	ND	ND	1.6	1.7	ND
HIS	5.4	6.9	5.0	6.5	5.8	8.4	5.9	7.0	6.5	5.0	9.7	4.0
LYS	10.4	10.4	8.7	8.8	8.7	13.5	10.5	10.2	10.4	8.9	13.3	9.8
ARG	8.4	9.3	7.6	9.0	9.5	6.7	9.6	9.9	7.6	6.7	8.9	7.8

Table 4: Amino Acid Composition of Apoferritin from Various Sources
Hu: Human; Ho: Horse; Ra: rat; Phyc: phycomyces; L: liver; S: spleen; H: heart; M: intestinal mucosa
From: Harrison, Clegg and May, 1980.

1978), comprise approximately 40% of the amino acid composition in most cases. The high leucine:isoleucine ratio is a characteristic of all the mammalian ferritins but this is less apparent in the phytoferritins. In addition the plant ferritins contain more aspartic acid and glutamic acid (or their amide forms) than the animal ferritins.

Using mainly manual sequencing methods, Heusterpreute and Crichton (1981) have been able to completely determine the primary sequence of horse spleen apoferritin. The result of their work is given in Figure 5. The amino acid sequence contains 174 residues and gives a molecular weight of 19824 for the subunit which is 7% greater than that found by physicochemical methods (Bryce and Crichton, 1971; Björk and Fish, 1971). The recent completion of the sequence together with the calculation of the electron density map at 2.8 Å resolution and the data obtained by X-ray crystallography (Banyard, Stammers and Harrison, 1978; Clegg *et al*, 1980) means that the three dimensional structure of horse spleen apoferritin may be determined in the very near future.

Apoferritin is a very compact molecule which is stable at elevated temperatures and only dissaggregates into its subunits at extremes of pH, with for example treatment with 66% (v/v) acetic acid at 0°C (Harrison and

Figure 5: The primary structure of horse spleen apoferritin as determined by Heusterpreute and Crichton, 1981.

Gregory, 1968). It is stable in 10M urea but dissociates in 6M guanidine hydrochloride and in 0.25% (w/v) sodium dodecyl sulphate at elevated temperatures (Smith-Johannsen and Drysdale, 1969). Circular dichroism and optical rotatory dispersion measurements have estimated the α-helical content to be approximately 66% (Listowsky *et al*, 1972) and this highly ordered secondary structure may explain the stability of the protein.

Due to the compactness of the molecule, the early electron density maps at 6 Å resolution could not clearly define the subunit boundaries, although rods of electron density were apparent and these were tentatively assigned as α-helices (Hoare, Harrison and Hoy, 1975). These features have been confirmed at 2.8 Å resolution and the subunit boundaries have now been delineated (Banyard, Stammers and Harrison, 1978).

Each subunit has been found to be roughly cylindrical with a height of 55 Å and a diameter of 27 Å and contained within each subunit are four long α-helices between 34 - 42 Å in length which lie parallel (or antiparallel) to the axis of the cylinder. In addition, there is a short region of α-helix which lies perpendicular to these 4 helices together with a length of extended polypeptide chain. A schematic drawing of a subunit of horse spleen apoferritin is given in Figure 6.

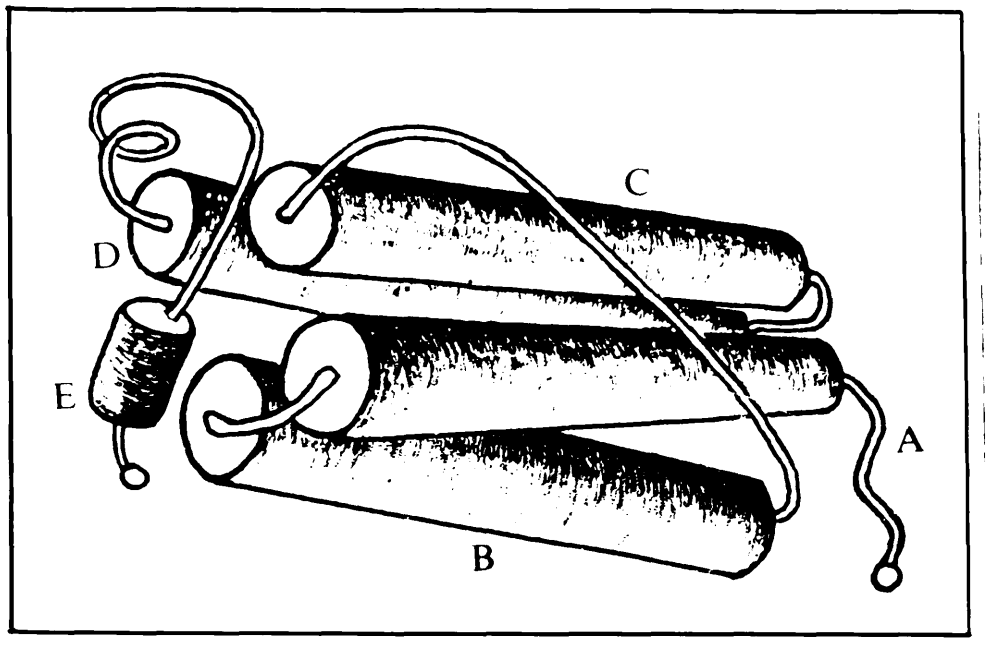


Figure 6: Schematic drawing of apoferritin subunit. The subunit contains four nearly parallel helices 34-42 Å long and a short helix E. The non-helical segments connecting the helices could not be assigned with certainty at this stage.

When the subunits are arranged in the protein shell the long helices are perpendicular to the radius vector forming a bilayer of helices around the central iron core, with one layer of helices facing inwards and the other outwards as shown schematically in Figure 7.

Weak regions in the electron density map at 2.8 Å resolution, where the interhelical connections occur, meant that the course of the polypeptide chain through the helical regions could not be traced unambiguously. Two different connectivities were possible (Clegg *et al*, 1980). Both of these start with the same N-terminal sequence of twelve amino acids which continue through helix A. It is at the C terminus of helix A where the two connectivities begin to differ since helix A may connect by a short turn to either the N-terminus of helix B or C. The helices are then subsequently joined as follows:

i) N → A → B → L → C → D → P → E

ii) N → A → C → D → P → E → B → L

with the alternative conformations that result being shown in Figure 8.

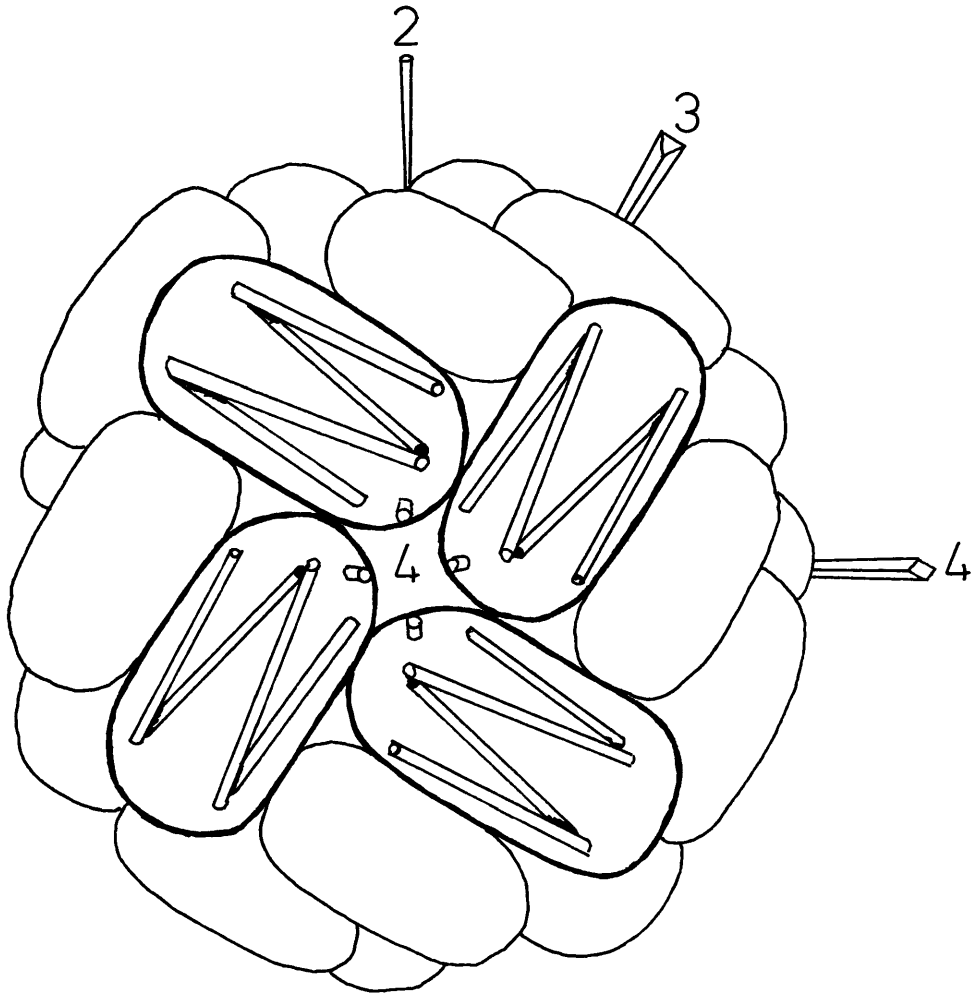


Figure 7: Diagram of the arrangements of helices within the apoferritin molecule. The 2-, 3, and 4-fold axes are also indicated.

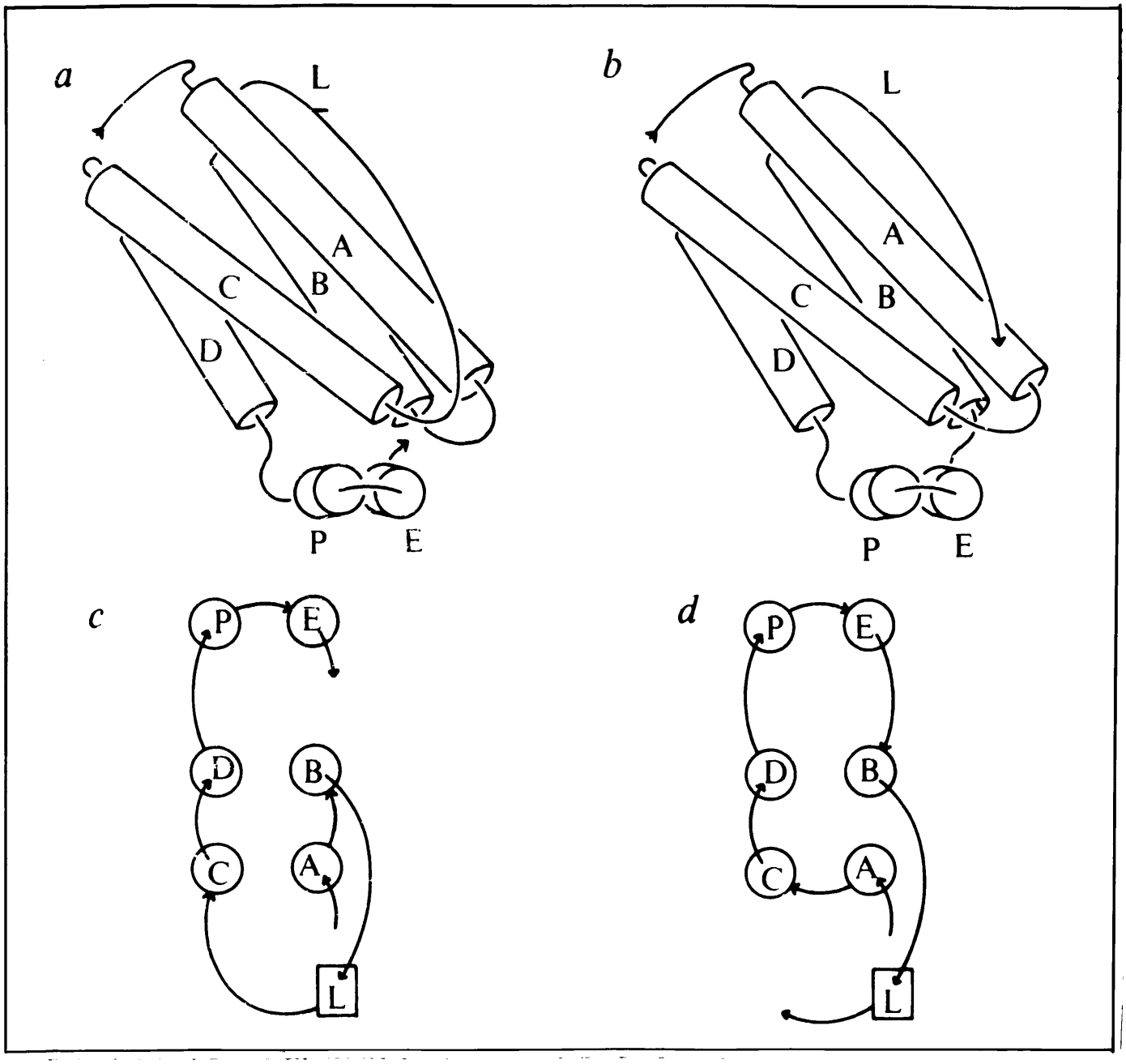


Figure 8: Alternative conformations of the apoferritin subunit.

(a) and (c) A-B-L-C-D-P-E

(b) and (d) A-C-D-P-E-B-L

With the recent elucidation of the complete primary sequence of horse spleen apoferritin (Heusterpreute and Crichton, 1981), Harrison has used a computer graphics system to fit the polypeptide chain residue by residue into the secondary structure. This together with predictions of the secondary structure from the sequence has allowed them to choose the first connectivity ABLCDPE (Bourne *et al*, 1982a). In this prediction the N-terminus is a turn of a 10 residues which connects with helix A (27 amino acids) on the external surface of the shell then turns into helix B (25 amino acids) which faces into the interior of the shell. The long loop L then connects the C terminus of helix B to the N-terminus of helix C on the outside of the shell partly covering helix A. Helix C (28 amino acids) on the external surface then turns into helix D (20 amino acids). The polypeptide chain then runs via the helical turn P through the short helical region E and ends with a non-helical tail inside the shell.

Packing of subunits into the quaternary structure of apoferritin suggests a pathway of assembly via dimers and hexamers (Bourne *et al*, 1982b) and the results of an earlier study would support this view (Crichton, 1972). Subunits related by 2-fold symmetry axes overlap along most of their length with two of the long helices from each subunit lying antiparallel to each other as can be

seen in Figure 9. A short stretch of antiparallel pleated sheet occurs near the 2-fold axis where the long length of extended chain (loop L) from neighbouring subunits are close enough to form hydrogen bonds. Four hydrogen bonds are formed involving a total of 5 residues in each subunit and the resulting pleated sheet lies on the outside surface of the molecule.

The dimer has a flat lozenge shape where the 8 long helices lie roughly parallel (or antiparallel) in two layers of four and overlap along most of their length. This may be a stable intermediate in the assembly of the 24-mer. Contact around the 3-fold axis is greater than around the 4-fold axis and the next stage in assembly may result from association of three pairs of dimers to form a hexamer.

Around the 3-fold axis the three dimers form a 3-blade propeller arrangement with the long axes lying roughly perpendicular to each other as is shown in Figure 10. The completion of the molecule could then occur by two of these hexamers associating to give a half molecule then step-wise addition of 2 hexamers or by interaction of 2-half molecules.

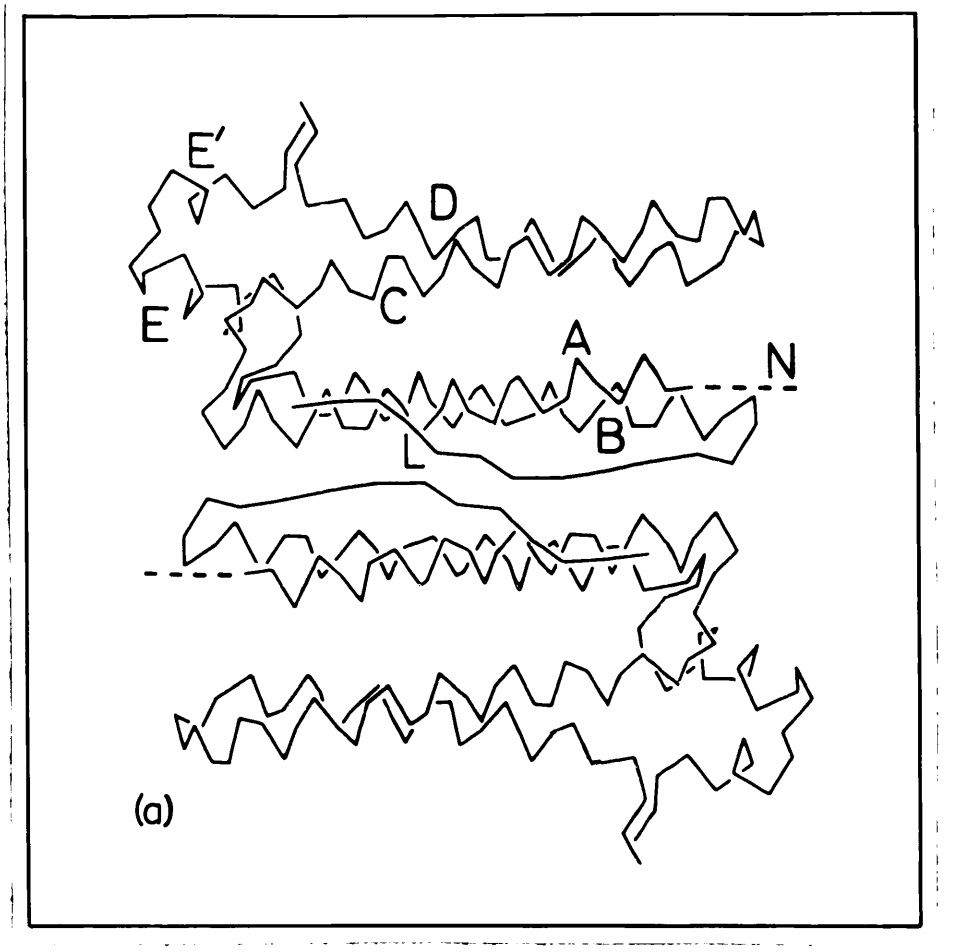


Figure 9: Dimer of two apoferritin subunits.
The drawing shows the course of the polypeptide chain of two subunits which are related by a 2-fold symmetry axis. The helices A, B, C, D and E are indicated.

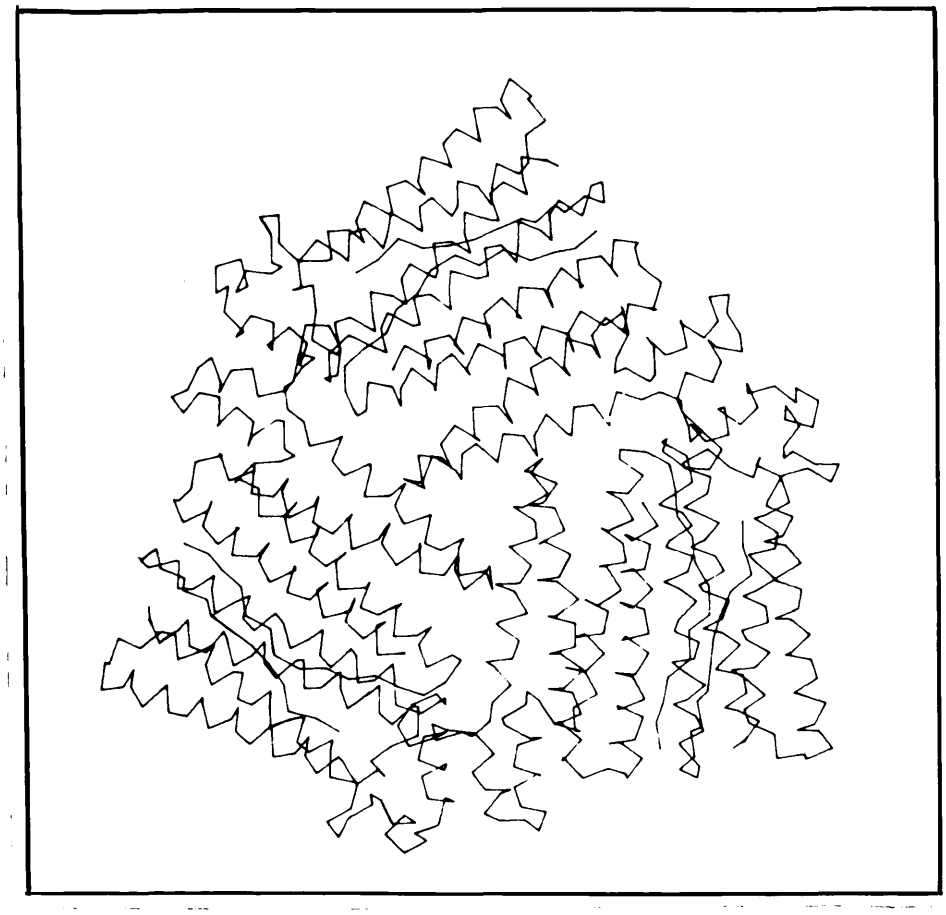


Figure 10: Hexamer of six apoferritin subunits.
The symmetrical hexamer formed from three subunit dimers is a proposed intermediate in apoferritin assembly.
From: Bourne *et al*, 1982b.

The tertiary structure of the subunit with its 4 long α helices lying roughly parallel to each other is a characteristic of several other proteins including cytochrome c, cytochrome b_{562}, tobacco mosaic virus, myohaemerythrin, and haemerythrin (Weber and Salemme, 1980). The angles between the helices in the apoferritin subunit closely resemble those in myohaemerythrin and the bundle of four helices in all these structures show a left-handed twist when viewed down their lengths, which may be considered as a stable 'super-secondary structure'. These structural relationships are shown in Figure 11.

There is relatively little subunit interaction in the region of the 4-fold axis and it is along the 4-fold axis where the channels which penetrate the shell occur. These channels are square in cross-section and are constricted in the middle by 4 short E helices, one from each of the four symmetry related subunits. The dimensions of these channels are 12 Å on the external surface widening to 16 Å in the interior of the molecule. The side chains of the E helices reduce the dimension at the centre of the channel to 7 Å as is shown in Figure 12. However there is a diagonal gap of 14 Å through which a flat molecule such as flavin could pass. Fitting the sequence to the tertiary structure suggests that the channels are

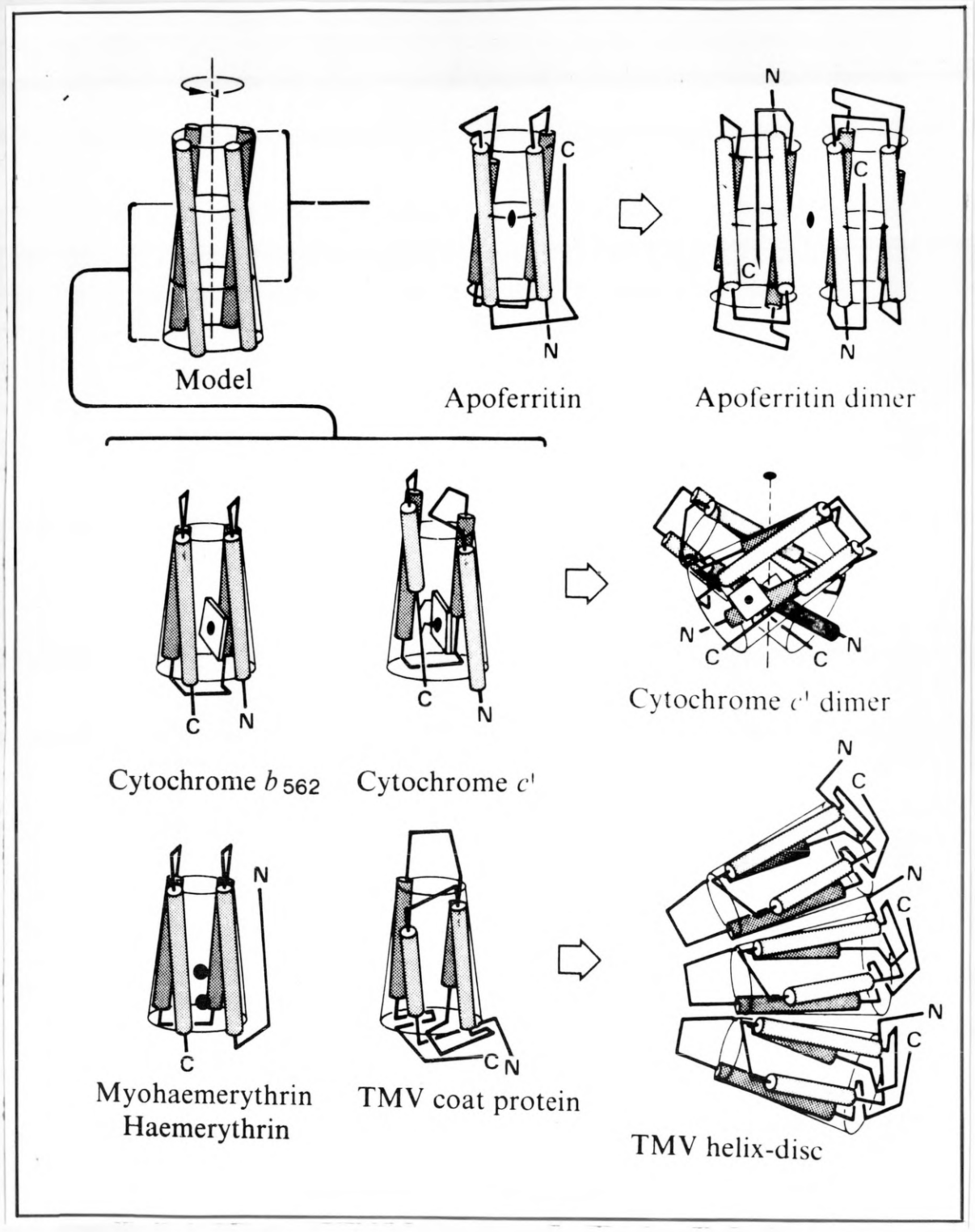


Figure 11: Schematic representation of the 4-α-helical proteins showing how they are related by a generalized 4-α-helical model.
From: Weber and Salemme, 1980.

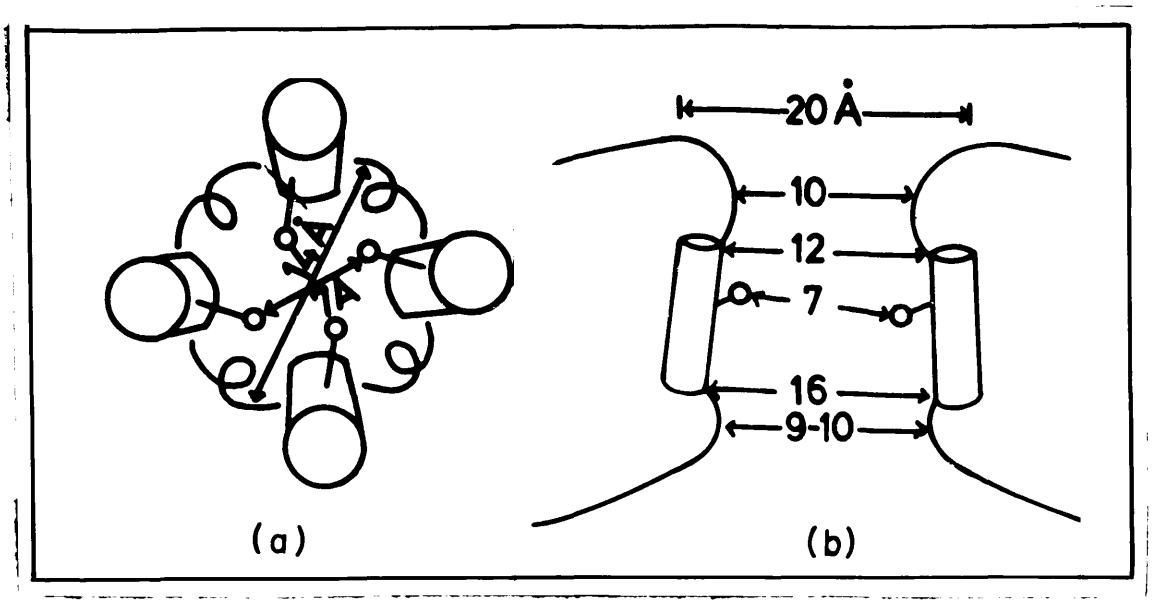


Figure 12: Diagrammatic representation of a channel through the apoferritin shell.

(a) 'End-on' view of channel seen from the inside of the molecule.

(b) 'Side-on' view, section of protein shell.

The cylinders represent helices and the 7 Å constriction made by a side chain can be seen in (b).

From: Harrison *et al*, 1978.

mainly lined with hydrophobic side chains. Detailed analysis of these channels may be of primary importance in understanding the mechanism of iron mobilization and deposition in ferritin.

Subunit Conformation and Chemical Modification

By applying the general principles of protein chemistry one might reasonably expect to find predominantly hydrophilic residues at the external and internal surface of the protein shell, with the hydrophobic residues lying inside the subunit structure. This would mean that one side of each of the long helices which is exposed to solvent would be hydrophilic and the other side of the helices would be hydrophobic. The hydrophilic sides of the helices would therefore be expected to be helices A and C on the outside surface and B and D where they face the internal cavity. This broadly agrees with the chemical modification data and the interpretation of the primary sequence.

In the subsections which follow the location of the various helical regions will be presented and the distribution and nature of their constituent amino acids discussed in terms of the overall three-dimensional structure.

Helix A

This helical region incorporates residues from glu^{11} to asp^{38}

^{11}glu-val-glu-ala-ala-val-asn-arg-leu-val-asn-

leu-tyr-leu-arg-ala-ser-tyr-thr-tyr-leu-ser-

leu-gly-phe-tyr-phe-^{38}asp

Helix A has a considerable number of interactions with helices B and C within the subunit and also with helix A' from a neighbouring subunit. This neighbouring subunit is symmetrically related to the first subunit and lies antiparallel to it. Helix A is also partly covered by the region of the polypeptide chain termed loop L.

The helix contains a high proportion of hydrophobic residues with four of the six tyrosine residues in the subunit being present in this section of the polypeptide chain. These four tyrosine residues, and also the tyrosine found in the N-terminal region at position 8, face inwards towards the subunit or at one of its interfaces. All of the charged groups in the helix are, as one would predict, on the external surface.

Helix B

This helical region incorporates residues from val^{47} to gln^{71} as is shown below:

^{47}val-cys-his-phe-phe-arg-glu-leu-ala-glu-glu-

lys-arg-glu-gly-ala-glu-arg-leu-leu-lys-met-

gln-asn-^{71}gln

Helix B contains a large number of acidic and basic groups and these lie either on or near the interior surface of the cavity. There is a distortion near the middle of this helix and this weakness in the helix has been attributed to the lysine residue at sequence No.58. The distortion is thought to occur because of an intrasubunit salt bridge which this lysine appears to form with a glutamate residue (No.103) from helix C.

Helix B also appears to form a number of other salt bridges but in these cases they are intersubunit salt bridges being formed with the B' helix of a diad related subunit and thus stabilizing the overall structure of the 24-mer. The residues which are thought to be in suitable positions to make these connections are glu (No.56), arg (No.59), glu (No.63) and arg (No.52) of helix B and arg' (No.59), glu' (No.56), arg' (No.52) and glu' (No.63) of helix B'.

Helix C

This helical region involves residues thr^{91} to ala^{119} and is shown below:

^{91}thr-thr-leu-asp-ala-met-lys-ala-ala-ile-val-leu-

glu-lys-ser-leu-asn-gln-ala-leu-leu-asp-leu-his-

ala-leu-gly-ser-^{119}ala

Helix C contains fewer charged residues than helix B and those it does contain point towards the interior of

the molecule. The glutamate residue at sequence No.103 is, as was mentioned earlier, involved in an intrasubunit salt bridge with the lysine of helix B at position 58.

Helix D

This helical region incorporates residues from his^{124} to lys^{143} as is shown below.

^{124}his-leu-cys-asp-phe-leu-glu-ser-his-phe-leu
-asp-glu-glu-val-lys-leu-ile-lys-^{143}lys

In helix D the charged residues appear to be mainly in contact with the solvent in the internal cavity and those which are orientated towards the interior of the subunit are thought to be involved in salt bridges. It contains one of the two cysteinyl residues present in the primary structure at position No.126 the other being present at the N-terminus of helix B. This helix is now thought to be longer than originally proposed because closer examination of the refined structure suggests that the electron density which was originally attributed to helix P is now thought to be part of helix D.

The two proline residues are both found in non-helical regions, one is present in the region of extended chain, namely loop L and the other is present in the short turn through which helix C connects to helix D.

Several chemical modification and titration experiments have been carried out to locate the position of certain amino acids within the tertiary structure of the subunit and also the 24-mer. The data obtained from these experiments agree well with the proposed structure of the subunit and the apoferritin shell and is summarized in Table 5.

Residue	Number in sequence	Availability to reagents	Probable position in 3D structure
lysine	9	Up to 7 reactive[ab] and titratable[c]	4 on external surface 3 on internal surface 1-2 in buried salt bridge 1 in surface salt bridge
arginine	11	Up to 10 reactive[a] and titratable	7 on external surface 4 on internal surface (4 of these may form salt bridges)
glutamate	15	Up to 12 reactive in apoferritin Up to 8 reactive in ferritin[b]	12 on or near internal surface 15 on or near internal surface (5 of these may form surface salt bridges)
aspartate	12	3 do not titrate[c]	
COOH		At least 2 protonated on subunit dissociation[a]	(-2 form buried salt bridges)
histidine	6	3 reactive and titratable[c]	3 on internal surface 3 buried in subunit or interfaces
cysteine	2	1-2 reactive[a,e]	1 on external surface 1 on internal surface
tyrosine	6	1 reactive[a,d] 5 transferred to solvent on dissociation[a,c]	1 on external surface 5 buried in subunit or interface.
tryptophan	1	tryptophan perturbed at pH 3.0 - 3.6[a]	trp on external surface partly exposed

a) Crichton and Bryce)1973)
b) Wetz and Crichton (1976)
c) Silk and Breslow (1976)
d) Macara (1974)
e) Hoare Harrison and Hoy (1975)

Table 5 Summary of chemical modification and titration data

From Bourne *et al* (1982a)

Isoferritins

The structural work which has been carried out on horse spleen apoferritin has led to a picture of the ferritin molecule being composed of 24 structurally and chemically identical subunits. Over the last ten years however, this premise has been brought into question with the development of more sensitive analytical techniques. It is now thought, by some workers, that there are two types of subunits in the protein shell although this hypothesis is one of the strongly contested areas of ferritin biochemistry and is actively being pursued in many laboratories.

Evidence for the presence of two subunit types has mainly come from two electrophoretic techniques namely isoelectrofocusing (IEF) and gradient-pore SDS electrophoresis. Richter in 1959 was one of the first workers to report differences in the electrophoretic mobility of ferritins isolated from normal tissues and tumour cells. Since then it has been observed that ferritins from different organs of the same animal may migrate at different rates in an electrical field (Lee and Richter, 1971; Linder *et al*, 1975). Ferritins from heart (Bomford *et al*, 1977) and bone marrow (Gabuzda, Rearson and Melum, 1969) were also found to have two distinct electrophoretic components. The term isoferritin

has been used to denote this difference in electrophoretic mobility. This may occur between ferritins from different species and between ferritins isolated from different organs of the same species.

The sensitivity of isoelectrofocusing has enabled ferritins from individual tissues, which would normally migrate as a single species on zone electrophoresis, to be resolved into a family of isoferritins. This multiple band pattern (microheterogeneity) has been observed with individual tissue ferritins from humans (Powel *et al*, 1975), horse (Drysdale 1977; Harrison *et al*, 1977) and rat (Lee and Richter, 1971; Urushizaki *et al*, 1973; Drysdale 1977). The number of isoferritins found so far from human, rat and horse tissues is about twenty but this figure is dependent on the type of commercial ampholyte used in the isoelectrofocusing analysis (Bredenkamp and Joubert, 1982).

That the microheterogeneity is real has been shown by trends in immunological reactivity and ion-exchange behaviour. Hazard *et al* (1977) found that antibodies raised against human liver ferritin gave reactivities in the order apoferritin > liver > heart > HeLa which corresponds to the electrophoretic trend of the isoferritins. Natural human apoferritin is electrophoretically the most basic of the human ferritins with heart ferritin followed by ferritin from HeLa cells being the most acidic.

This trend however is reversed in the case of horse ferritins where ferritin isolated from heart tissue has been found to be more basic than either the spleen or liver ferritins.

In order to explain the origin of this microheterogeneity Drysdale and co-workers have produced a model which is based on the results of experiments using isoelectrofocusing and gradient-pore SDS electrophoresis. When a sample of ferritin is subjected to SDS electrophoresis it reveals the presence of a single subunit band and some additional minor bands with lower molecular weights. However, just as isoelectrofocusing is a much more powerful technique in terms of resolution than zone electrophoresis gradient-pore SDS electrophoresis has a higher resolving power than electrophoresis in SDS gels of uniform porosity. Using this technique Drysdale and colleagues have been able to show the presence of two types of subunits in ferritin which differ in molecular weight (Adelman, Arosio and Drysdale, 1975; Arosio, Adelman and Drysdale, 1977). These two subunits have molecular weights of 19,000 and 21,000 and have been designated L or light subunit and H or heavy subunit respectively. Drysdale proposes that ferritin is formed as a hybrid of these two subunits giving a possible twenty five different ferritin molecules whose composition range from $L_{24}H_0$ to L_0H_{24} as shown in Figure 13. The observed microheterogeneity could

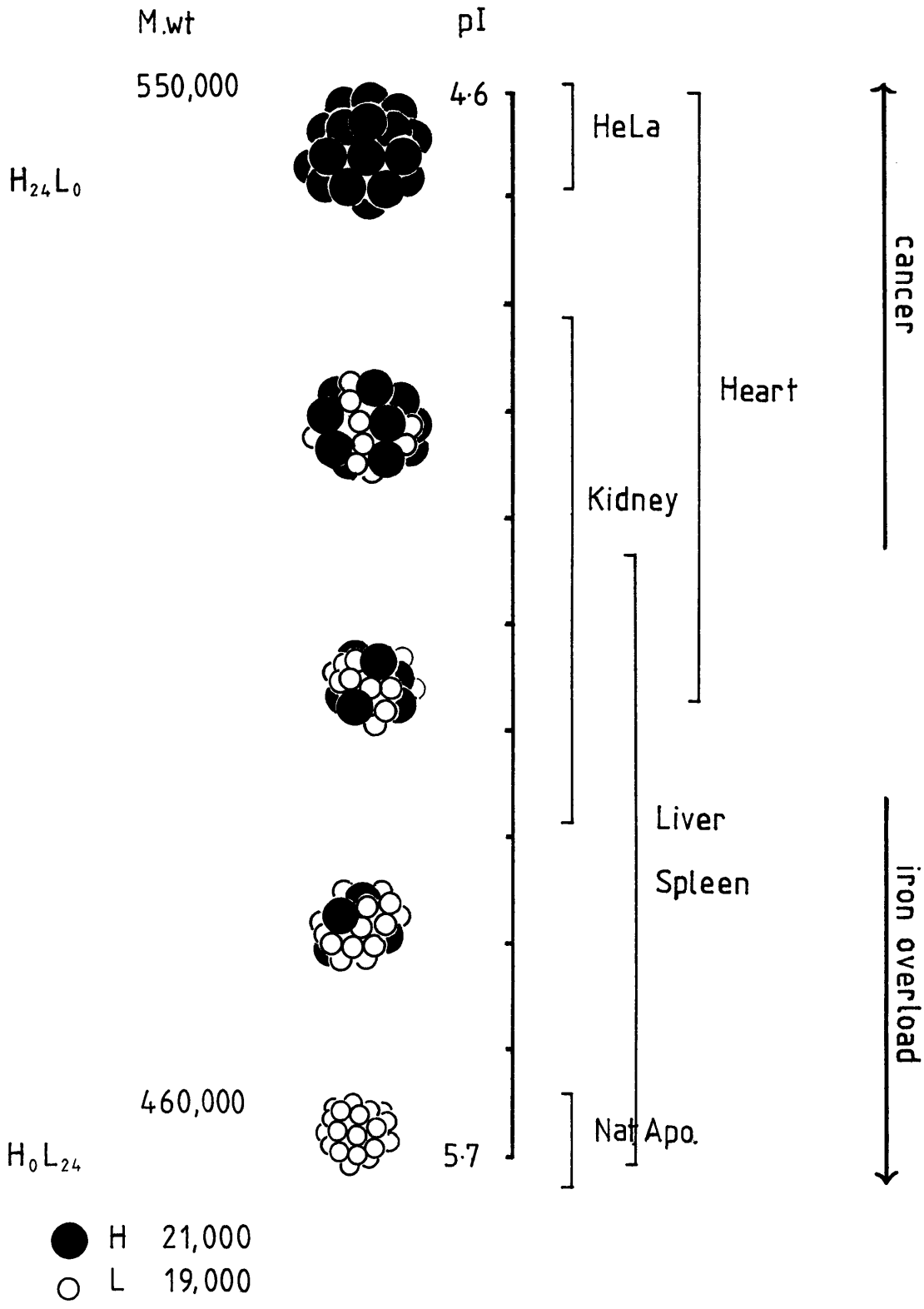


Figure 13: Drysdale's Model of human isoferritins.

then be explained by slight differences in the electrophoretic behaviour of the two subunits. The functional significance of the two subunits is unclear although the most obvious reason would be to influence the rate of iron uptake and release.

Russel and Harrison (1978) and Wagstaff, Worwood and Jacobs (1978) have both found a rough correlation between iron content and pI. When ferritins from human liver and spleen were fractionated with respect to iron content they found that as the pI of the ferritin increased the iron content also increased. If the iron was then removed from the molecules and allowed to be re-incorporated then those molecules which originally contained the least iron had the slowest rate of uptake. When horse liver and spleen were examined a trend was again apparent but in this case the pI appeared to decrease with respect to iron content. Not all workers have been able to correlate iron status with isoferritin patterns, Treffry and Harrison (1980), using rat liver ferritin found that it was the initial amount of iron present in the ferritin which determined the rate of iron uptake into the apoferritin *in vitro* and not some property of the protein alone (such as subunit composition).

As mentioned previously, when ferritin is electrophoresed in the presence of SDS some protein bands, in addition to

those of the subunit, are visible. These bands have been found to have molecular weights of 7,500 and 11,000 and are thought to have arisen as the result of a specific proteolytic cleavage of the subunit during its isolation procedure. The incorporation of the protease inhibitor phenylmethylsulphonylfluoride during the isolation of ferritin significantly reduces the appearance of these peptides in SDS gels. In the absence of any thiol reagent additional bands are seen. Several bands are visible with molecular weights higher than that of the subunit and are thought to be dimers and tetramers of the subunit associated via intra-molecular disulphide bonds. The presence of an additional band whose molecular weight is 15,000 is often observed in the absence of a thiol reagent and it has been suggested that this represents the subunit with an intramolecular disulphide bond giving an apparent smaller molecular weight. Addition of thiol to the sample prior to electrophoresis causes this band to decrease with a concomitant increase in the 19,000 molecular weight band (i.e. the subunit) (Ishitani, Niitsu and Listowsky, 1975).

It has been noted by several workers that tissue ferritins contain carbohydrate (Shinjyo, Abe and Masuda, 1975; Cynkin and Knowlton, 1977). Cynkin and Knowlton, in an extensive study of horse spleen, human liver and heart ferritins have shown that sugars are present on

isolated subunits which confirms that the presence of carbohydrate is not due to contamination. They have found that human heart ferritin (predominantly H subunits by Drysdale's model) contains nearly twice as much sugar as liver or spleen ferritins (predominantly L subunits). The difference in carbohydrate content is not sufficient to account for the different subunit sizes but may influence the isoelectrofocusing profile. Covalent incorporation of sugars into ferritin has been shown to occur non-enzymatically. Zaman and Verwilghen (1981) found that the incorporation of sugars occurred in the order glucose $\geq$ mannose > fucose but that the degree of attachment depended on the concentration of ferritin, sugar and length of incubation. They have proposed that the covalent attachment of sugar occurs via free amino groups available on the protein and this blocking of the amino groups could explain the occurrence of isoferritins. It has been found that carbohydrate side chains play an important role in regulating the catabolism of glycoproteins (Ashwell and Morell, 1974). However, Zaman and Verwilghen found glycosylation of ferritin had no effect on the plasma half-life of rat liver ferritin and also did not effect its immunological properties. Glycosylated ferritin has also been detected in human serum (Cragg, Wagstaff and Worwood, 1981).

Additional evidence against the microheterogeneity observed in isoelectrofocusing has come from several workers. Lee and Richter (1971b) and Bryce and Crichton (1973b) have suggested that some of the isoferritins observed may be artifacts due to an interaction between the ferritin molecules and various components in the gel notably traces of the strong oxidant ammonium persulphate and also binding of the protein to the carrier ampholyte used to establish the pH gradient in isoelectrofocusing. Shinjo and Harrison (1979) have shown that the discrete bands which are obtained in isoelectrofocusing experiments are due to discrete bands in a discontinuous ampholyte pH gradient rather than individual isoferritins. As mentioned previously it has also been shown that the multiple band pattern obtained depends on the commercial ampholytes used in the experiment (Bredenkamp and Joubert, 1982). It has also been suggested that the microheterogeneity could arise through surface charge modifications of the protein during the rigorous isolation procedure. (Richter, 1975; Collet-Cassart and Crichton, 1975). An exposed methionine in the N-terminal region of the primary structure has been suggested by Collet-Cassart and Crichton as a possible residue susceptible to oxidation and thus introducting additional negative charges to the surface of the protein. The heat-step used in the isolation procedure for ferritin has also been proposed as a source

of artifacts but this view has since been discounted as ferritins isolated without any heat-step also display multiple bands on isoelectrofocusing (Worwood *et al*, 1976; Lavoie, Ishikawa and Listowsky, 1978.) Finally it has also been argued that the presence of multiple forms of ferritin in a single tissue is due to several cell types. Brown and Thiel (1976) however were able to show that ferritin isolated from a uniform population of tadpole red cells showed microheterogeneity.

Two different subunits from human liver have been isolated by a process involving electrophoresis then gel filtration in denaturing conditions (Otsuka, Maruyama and Listowsky, 1981). Circular dichroism studies on the reassembled H homopolymers (i.e., 24 subunits) and L homopolymers indicate that the two subunits are conformationally distinct. The L subunit having a more ordered structure, with approximately 50% α helical content, than the H subunit whose α helical content has been estimated to be 30%. The fluorescence spectra of H and L homopolymers suggest that both subunits contain tryptophan but that the environment in which the tryptophan is found differs in the two subunits. It has been suggested that the L subunit is formed from the H subunit by post-translational modification. However, whilst the amino acid composition of the H and L subunits are similar the presence of fewer leucine, phenylalanine, and arginine

in the larger H subunit makes it improbable that H is the precursor of L and hence it is assumed that they arise as the products of separate genes.

Structural studies on horse spleen apoferritin monomer have shown it to be a highly ordered structure. It is difficult to envisage how a subunit containing an extra twenty amino acids could fit into such a structure especially if the subunit differs significantly in its conformation. If the extra amino acids are present at the C-terminal region then the additional amino acids will be located in the internal cavity of the protein. However, H-rich ferritins are generally assumed to be iron rich which would make it unlikely that the extra amino acids are located internally. The N-terminal region is on the external surface of the protein shell so that additional amino acids may be accommodated here. The evidence for the difference in molecular weight of the two subunits has been based on gradient-pore SDS electrophoresis but not all the data are consistent. If the estimations of molecular weight differences have been exaggerated then it may be possible that the two subunits have arisen from amino acid substitution rather than addition. If the differences in amino acids were to occur on the surface of the protein then this would explain the results of techniques based on electrophoretic mobility and antigenicity. If this were the case then the

regions of subunit-subunit interaction would be likely to be conserved and the concept of a hybrid molecule would be more plausible.

Biological Function of Iron

The function of ferritin is to provide the body with a readily available source of iron. The structure of this soluble molecule enables the organism to obtain ferrous ions when required for its metabolic processes without being exposed to the harmful effects of free iron. Any excess iron can be stored within the ferritin molecule until required at a later date. The storage of iron is therefore a dynamic one with iron constantly being deposited or released from the ferritin molecule.

In 1955, Bielig and Bayer demonstrated that a ferritin-like molecule could be formed by the addition of $Fe(NH_4)_2(SO_4)_2$ to apoferritin, the protein moiety of ferritin in the presence of air (Bielig and Bayer, 1955). Since this time the mechanism of ferritin formation has been the subject of many studies. However neither the physiological processes leading to the deposition of iron into ferritin or the mechanism for releasing iron are, as yet, fully understood.

Deposition of Iron

In 1968 it was suggested by Pape *et al* (1968) that ferritin is formed by the subunits assembling around pre-existing iron cores. This hypothesis is supported

by the fact that certain ferric salts may form inorganic polymers which are spherical in shape and are about 70 Å in diameter which is approximately the same molecular dimension as the iron micelle core of ferritin. An alternative model for ferritin formation, which is currently favoured by most workers, has been proposed. In this model iron is able to enter the hollow protein shell, presumably through channels, and polymerize inside the central cavity, with release of iron occurring via the same channels. This latter mechanism is supported by observations that ^{14}C labelled amino acids are incorporated initially into apoferritin or iron-poor molecules during iron-induced ferritin synthesis (Listowsky *et al*, 1972).

Ferritin stores iron as ferric oxyhydroxide but accepts it as the ferrous form. Several workers have been able to show that apoferritin accelerates the rate of oxidation of Fe(II) to Fe(III) *in vitro* with a concomitant formation of aggregated ferric oxide inside the protein shell (Bryce and Crichton, 1973; Macara, Hoy and Harrison, 1972; Neiderer, 1970).

The mechanism of iron uptake has been studied both *in vivo* (Hoy and Harrison, 1975) and *in vitro* (Macara, Hoy and Harrison, 1973; Harrison *et al*, 1978) and in both cases it has been shown that ferritin molecules which

are only partially filled take up iron more readily than apoferritin. This effect can be seen in Figure 14 where initial rates of iron uptake were measured either with successive additions of iron or single additions to fractions of different iron content at the same protein concentration (Harrison *et al*, 1978). It can be seen that the presence of a low concentration of iron within the molecule accelerates the rate of iron uptake, but this effect starts to decrease once the concentration of iron within the molecule reaches a certain level.

The pattern of growth of the iron micelle can vary from sigmoidal to hyperbolic (Crichton and Bryce, 1973). The type of curve obtained depends on several factors including pH, the iron and protein concentration and the nature of the buffer used. At pH 7,in buffers which do not strongly chelate, iron progress curves are hyperbolic at low iron/protein concentration and become sigmoidal as the iron/protein concentration is increased (Macara, Hoy and Harrison, 1972). At low pH and in strongly chelating buffers, the progress curve is again hyperbolic (Paques and Crichton, 1979). The sigmoidal curve suggests two phases in ferritin formation, these being an initial slow phase followed by a second phase where iron incorporation is accelerated. These observations have led Macara to propose a 'crystal growth' model of

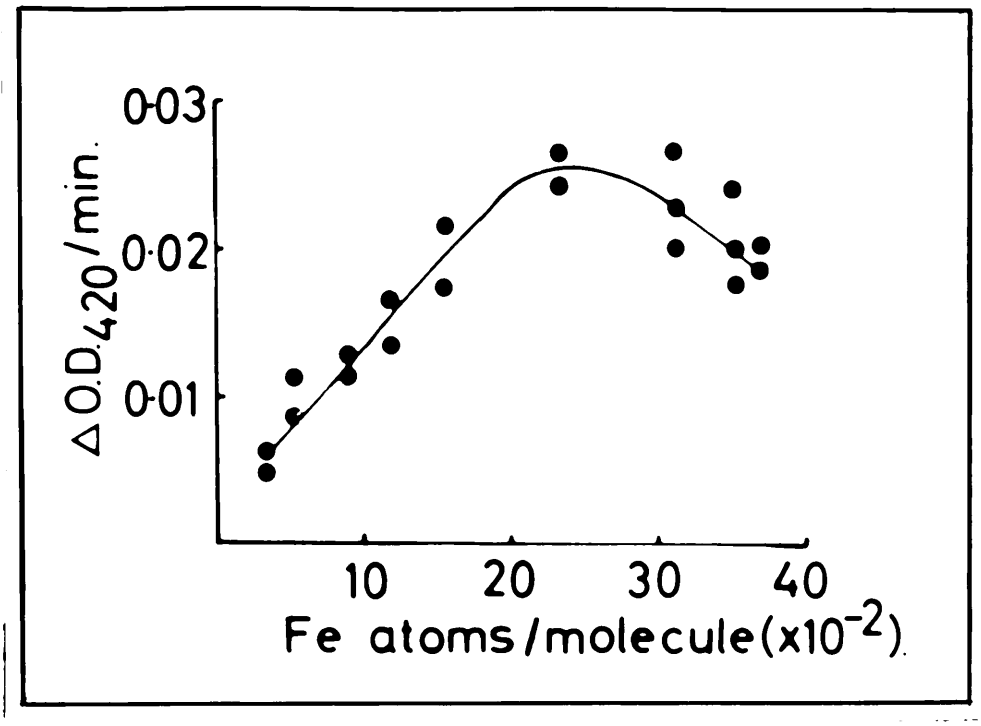


Figure 14: Initial rates of iron uptake by horse spleen ferritin fractions at constant protein concentration. From Harrison *et al* (1975).

ferritin formation (Macara, Hoy and Harrison, 1972; Harrison and Hoy, 1973).

In this model iron enters the protein shell via the six channels which allow the passage of small molecules into the central cavity. On the inner surface of the shell are several catalytic sites which are able to catalyse the oxidation of Fe(II) to Fe(III) in the presence of an oxidant. At these catalytic sites the Fe(III) which is formed is deposited and provides a nucleation centre for the growth of microcrystals of ferric hydroxide. This nucleation stage is a relatively slow stage however, once formed, iron is able to be directly oxidized by, and added to, the growing crystal without the need to pass through specific oxidation sites on the surface of the protein. This results in an accelerated rate of iron uptake. As the crystal grows both the channels through the shell and the catalytic sites begin to be covered over by the deposited iron. This together with the fact that the surface area will begin to decrease due to the confines of the protein shell, results in a decrease in the rate of iron uptake by the ferritin molecule. This pattern of growth can be seen in Figure 15.

The presence of several catalytic sites inside the molecule provides centres for the formation of several nucleation sites. However, it is thought that the first

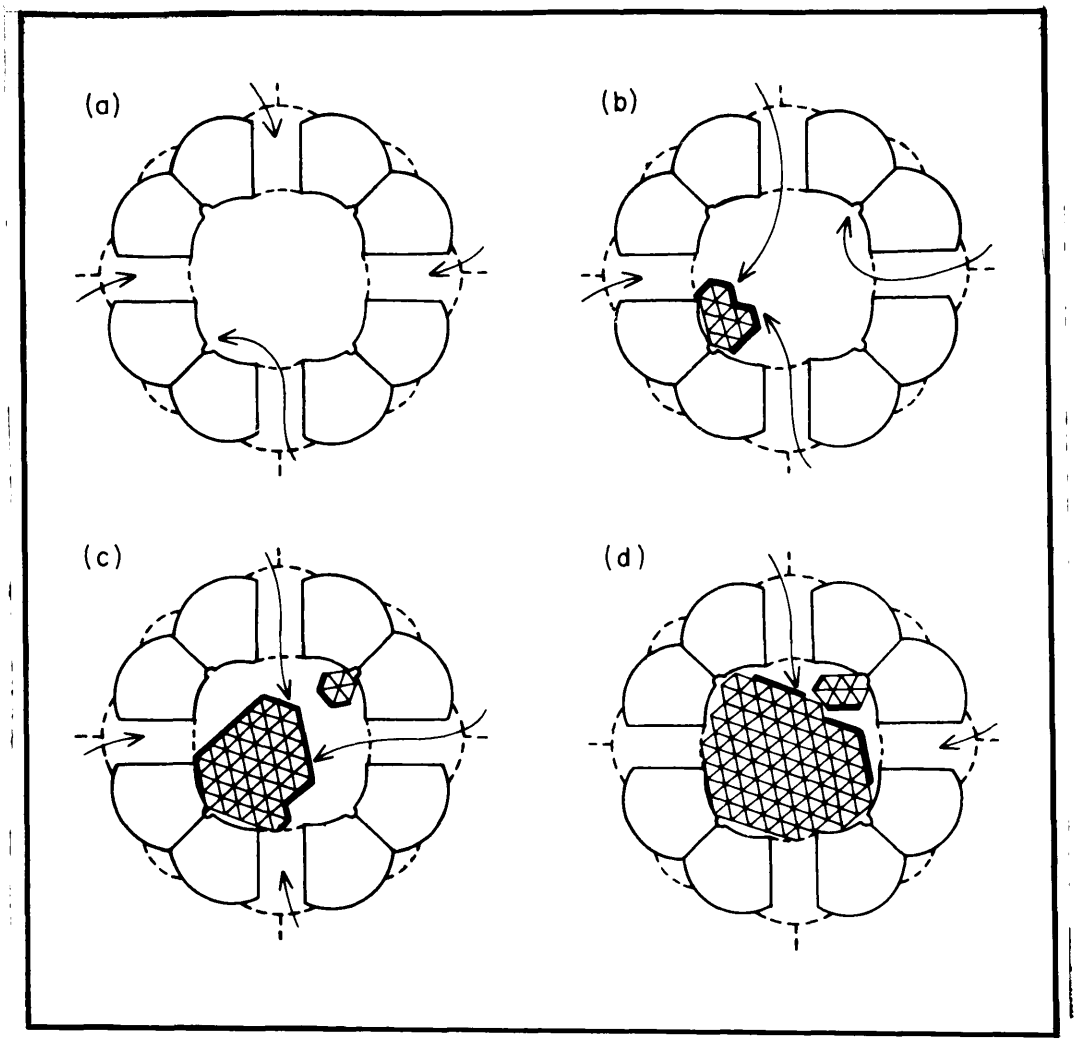


Figure 15: A model for ferritin iron uptake and release.

a) Fe(II) enters the shell through channels and is bound at sites favouring oxidation.

b) An Fe(III) OOH iron-core nucleus forms on the bound Fe(III).

c) and d) The microcrystal builds up by oxidation of further Fe(II) at its surface (another nucleus may also form).

Available surface for Fe deposition (thick line) first increases and then decreases as the molecule fills.

Iron is lost from the microcrystal surface so the last added iron is released first.

nucleation site formed grows at the expense of the others, so that under conditions of rapid crystal growth only a single crystal is observed. This scheme is illustrated in Figure 15(c) and (d).

An alternative mechanism has been proposed by Crichton and colleagues which involves separate sites for oxidation and heteronucleation (Crichton *et al*, 1977; Crichton and Roman, 1978). In this model oxidation sites are located in the channels penetrating the shell. This means that they are permanently accessible to incoming iron so that all the iron entering the molecule may be oxidized by these sites. Two ferrous ions are involved at the oxidation site and they are oxidized by dioxygen to give a peroxocomplex in which the O_2^{2-} anion is held between two ferric atoms. This complex is then hydrolyzed to form ferric oxyhydroxide which is displaced to nucleation sites in the central cavity by incoming ferrous ions. This displacement is a result of the oxidation sites having a higher affinity for the ferrous ions than ferric ions. The process is shown schematically in Figure 16. The last stage is thought to be reversible so that iron is able to equilibrate between the heteronucleation sites and oxidation sites, with the direction of iron flux being dependent on the concentration of iron in the surrounding medium.

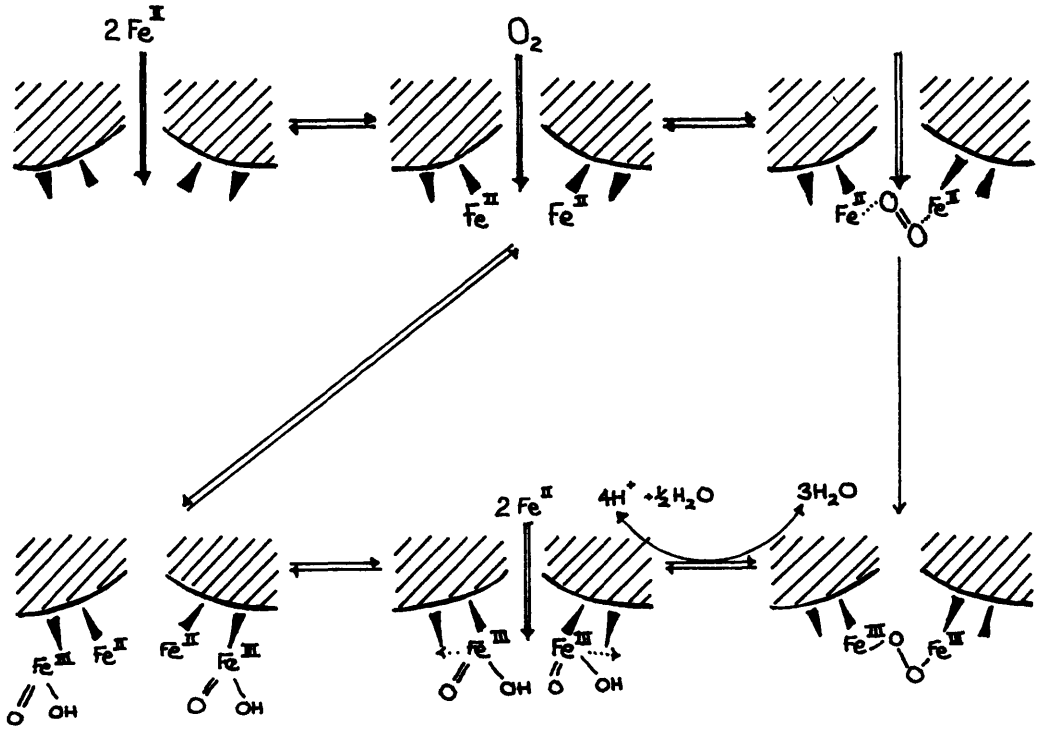


Figure 16: Alternative mechanism of ferritin formation involving specific Fe II oxidation sites.

There is no direct evidence which favours either of the two models, however, there is certain experimental data which would be difficult to explain with the model of Crichton. In his model the sites for binding Fe(II) must be permanently available in order for iron deposition to occur. Harrison *et al* (1978) has shown that when these sites are blocked by modification or by Zn(II), iron accumulation can still occur provided an iron-core nucleus is present.

Wetz and Crichton (1976) have shown that carboxyl groups are involved in the deposition process. These workers modified carboxyl groups with glycineamide and carbodiimide in both ferritin and apoferritin. In apoferritin eleven carboxyl groups could be modified and the resulting apoferritin was unable to accumulate iron. In full ferritin four of these eleven carboxyl groups were protected. If iron was then removed from the molecule the modified apoferritin which was produced was able to incorporate iron with the same ability as unmodified apoferritin. This suggests that the carboxyl groups which are essential for catalysis must be on the inner surfaces of the protein shell.

Experiments involving equilibrium dialysis with zinc have revealed the presence of both high and low affinity sites for Zn(II) (Harrison *et al*, 1978). If the zinc

concentration is such that the high affinity sites are saturated then there is almost a complete inhibition of iron uptake by apoferritin but only 50% inhibition of iron uptake by ferritin. This finding gives further support to the model proposed by Macara.(Macara, Hoy and Harrison, 1972).

Release of Iron

The way in which cells remove iron from ferritin is still uncertain, however, it is generally agreed that intracellular release of iron occurs from intact ferritin molecules without requiring their degradation or dissociation. The process of iron release must involve reduction and iron may be mobilized by dithionite and thioglycollate in the presence of a chelating agent such as EDTA, desferroximine or o-phenanthroline. The chelating agents themselves are ineffective in removing iron or remove it only very slowly. Some intracellular reducing agents such as cysteine, glutathione, and ascorbic acid are also capable of mobilizing iron but their rate of iron release is not sufficient enough for them to be considered as physiological mediators of iron release. In contrast it has been shown that iron is rapidly and quantitatively released by incubation with $FMNH_2$, $FADH_2$ and reduced riboflavins at physiological pH (Sirivech, Frieden and Osaki, 1974). In order for these molecules

to interact with the protein core they must be able to negotiate the channels through the protein shell. The existence of six channels along the 4-fold axis has been known for some time but recent evidence from Harrison and colleagues suggests the possibility of an additional eight channels which lie along the 3-fold axis (Harrison, 1983). The dimensions of either or both of these types of channels may be a rate-determining step in the release of iron from the molecule, however, it is not known how flexible the protein is in solution.

Experiments by Walsh and colleagues have shown that reduced flavins exhibit saturation kinetics in the reductive release of iron from horse spleen and horse heart ferritins (Jones, Spencer and Walsh, 1978). When they examined flavins with different reduction potentials they found that each flavin had a different V_{max} and that those flavins with the most thermodynamically favourable driving force had the fastest V_{max} for the reductive release of iron. This correlation would suggest that it is the redox step and not the diffusion of reductant into the ferritin core which is the rate determining step.

That the dehydroflavins must penetrate the channels to effect Fe^{2+} release has been shown by the fact that reduced flavins which have been covalently bound to Sepharose

are ineffective in releasing iron from ferritin but are able to reduce iron cores when they are free in solution (Jones, Spencer and Walsh, 1978). This would support evidence that the reduced flavins must traverse the protein shell to be effective in causing release of Fe(II) from the iron core.

Iron is thought to be mobilized on a first-in-last-out principle. In the mechanism of iron-core formation proposed by Macara and co-workers, it is believed that iron cores are built up by the incoming iron being deposited on the surface sites of the iron micelle (Macara, Hoy and Harrison, 1972). Therefore, in the case of iron mobilization one would also expect iron to be mobilized first from the surface layers of the iron core since this iron is more available to reagents than iron in internal positions. Experiments carried out by Hoy *et al* (1974) and Treffry and Harrison (1979) would support this first-in-last-out principle. These workers found that if ^{59}Fe (either as Fe(II) or Fe(III)) was added *in vitro* to ferritin molecules with a low iron content and then the iron was slowly allowed to be released,the labelled material was predominantly found in the fractions of iron which were mobilized first.

The properties of ferritin allow it to take up excess free iron in the cytoplasm and release this stored iron when demanded by the cells metabolism. In order to provide

a reserve capacity for rapid removal of free iron in the cytoplasm, it is advantageous to have ferritin molecules with a low iron content. The kinetics of iron uptake, which are optimal at relatively low saturation ensure a rapid uptake of iron into molecules with a low iron content. Release of iron from ferritin by reducing and chelating agents also varies with the iron content of the molecule, being most efficient with molecules that are relatively unsaturated (Crichton, Wauters and Roman, 1975). Thus under conditions of intense iron storage, the retained iron will be less easily mobilized than from ferritin in a subject with relatively low iron stores and partly unsaturated ferritin shells, a physiologically desirable feature.

MATERIALS AND METHODS

MATERIALS AND METHODS

Isolation Procedures

In this study commercial horse spleen ferritins were obtained from Miles-Pentex Corporation, Boehringer-Mannheim and Serva Feinbiochemica, Heidelberg. The preparations of horse and human ferritins were kindly supplied by J.C. Maréschal (Louvain-la-Neuve, Belgium) or prepared in the laboratory using the method of Crichton *et al* (1973) as outlined in Figure 17.

The protease inhibitor, phenylmethylsulphonyl fluoride (PMSF), was incorporated into the isolation procedure at a concentration of 2mM to reduce the degree of proteolytic activity.

Apoferritin was prepared from ferritin by dialysis against two changes of 1% (v/v) thioglycollic acid in sodium acetate buffer pH 5.5. The apoferritin produced in this way was then dialyzed extensively against 1mM Tris buffer pH 7.2. In some cases the protein was further desalted by passing through a Sephadex G-25 column equilibrated with 10mM Tris buffer pH 7.2.

Protein Determinations

Protein concentrations were determined using $E^{1cm}_{1\%}$ 280nm values of 0.981 (for horse spleen), 0.974 (for

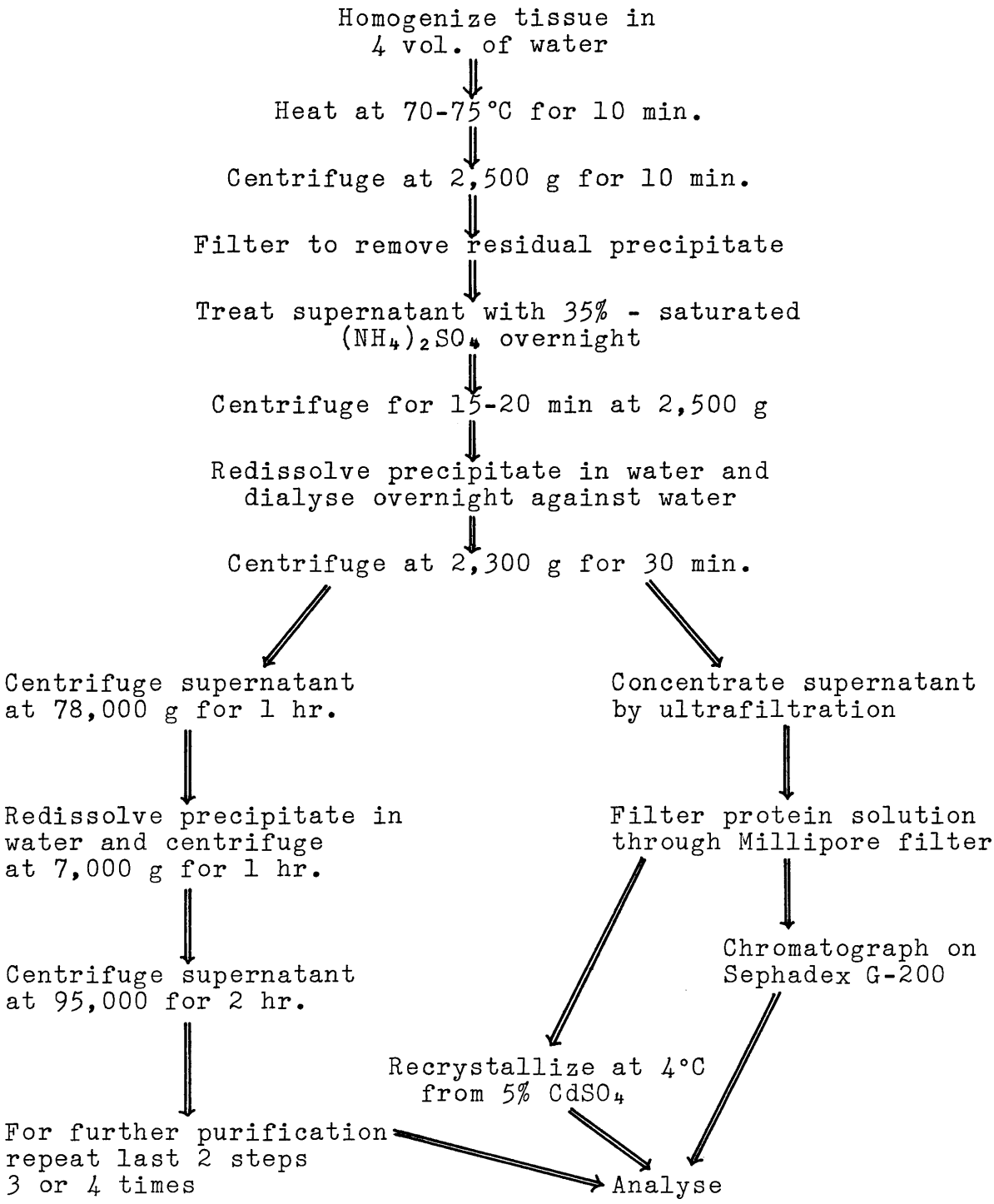


Figure 17: Isolation procedures employed for preparation of ferritin.

human spleen), 0.911 (for horse liver), and 1.043 (for human liver). These were calculated from the amino acid compositions (Crichton, Millar, Cumming and Bryce, 1973; Crichton *et al*, 1979) of the various ferritins using a refined integer fit technique (Bryce, 1979) and the equation employed by Edelhoch (Edelhoch, 1967).

Protein concentrations were also estimated using the Bio-Rad protein assay procedure as described below.

Both the standard and microassay procedures were used. The standard assay procedure is sensitive in the range 200-1400µg/ml whilst the microassay is sensitive in those instances where the concentration of protein is less than 25µg/ml.

Standard Assay Procedure

Several dilutions of bovine serum albumin (Sigma) containing 0.2 to 1.4 mg/ml were prepared.

0.1 ml of the standards and appropriately diluted samples were placed in test tubes.

0.1 ml of sample buffer were used as a blank.

5.0 ml of the diluted dye reagent (1 in 5 dilution of Dye Reagent Concentrate with water) were added to each tube.

The tubes were vortexed and left for a period of 5 minutes to 1 hr after which the absorbances at 595nm were measured against the reagent blank.

Microassay Procedure

Several dilutions of bovine serum albumin containing from 1 to 25µg/ml were prepared.

0.8 ml of the standards and appropriately diluted samples were placed in test tubes.

0.8 ml sample buffer were used as a blank.

0.2 ml Dye Reagent Concentrate were added. to each tube.

The tubes were vortexed and left for a period of 5 minutes to 1 hr after which the absorbances at 595nm were measured against the reagent blank.

Separation of Glycosylated and Non-glycosylated Horse Spleen Apoferritin

The presence of a glycosylated subunit in horse spleen apoferritin was investigated using an affinity column for glycoproteins. Glyco-gel B (Pierce) contains boronate ligands immobilized on cross-linked beaded agarose.

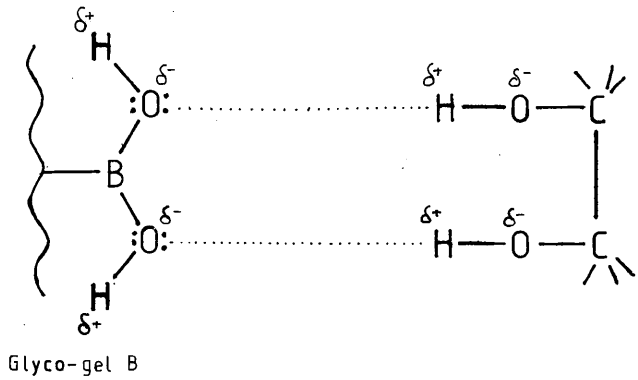


Figure 18: Structure of Glyco-gel B matrix.

To be retained on the Glyco-gel B matrix the glycoprotein must contain a sugar moiety which presents a vic-diol to the immobilized boronic acid.

This support medium was used to separate glycosylated ferritin from non-glycosylated ferritin.

5 ml of the support material were equilibrated with 0.25M ammonium acetate, pH 8.5. Using a flow rate of 0.5 ml/min, 300µl (15 mg) horse spleen ferritin (Boehringer Mannheim) were applied to the column. The non-glycosylated material was eluted from the column with 0.25M ammonium acetate pH 8.5. 1% (v/v) 2-mercaptoethanol was then incorporated into this buffer to elute non-glycosylated ferritin which remained attached to the column via a disulphide linkage to a glycosylated subunit. After this fraction was eluted the eluant was changed to 0.25M ammonium acetate, pH 8.5 in order to re-establish the baseline on the recorder due to the absorbance of 2-mercaptoethanol at 280nm. Glycosylated ferritin was eluted with 0.2M sorbitol pH 8.5. The column was

regenerated with 0.1M acetic acid followed by 0.1M HCl. The small volume fractions obtained were dialyzed against 1mM Tris pH 8.8 and concentrated by surrounding the dialysis sacs with Sephadex G-200. Using this method fluid is withdrawn from the dialysis sac by the Sephadex beads while protein is retained within the dialysis sac by the dialysis membrane.

Column Chromatography in 6M Guanidine Hydrochloride

Sephacryl S-200 superfine was chosen as the medium to separate horse spleen apoferritin subunits from peptide fragments. Because of its chemical stability, Sephacryl may be used with eluents such as 6M guanidine hydrochloride and its mechanical strength allows relatively high flow rates to be used even in large preparative columns.

Sephacryl S-200 superfine was commercially prepared by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide to give a rigid gel with a carefully controlled range of pore size. A hypothetical partial structure is shown overleaf:

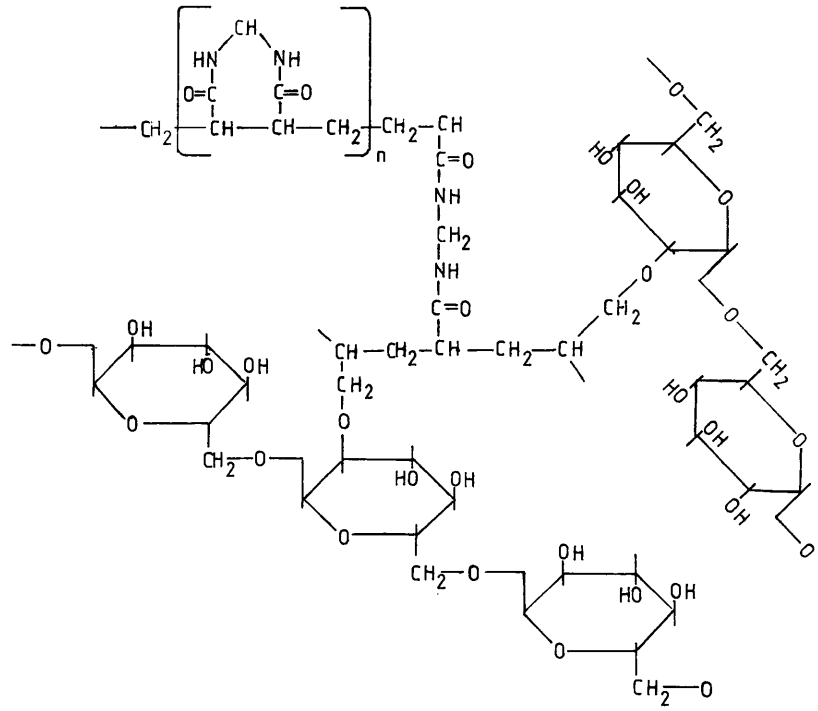


Figure 19: Partial structure of a Sephacryl S-200 superfine gel

The wet bead diameter is 40-105μ.
Using flow ratesof up to 30 ml/hr proteins in the molecular weight range 5,000 - 250,000 may be separated.

A column of Sephacryl S-200 superfine (95 cm x 2.5 cm) was equilibrated with 6M guanidine hydrochloride. 50 mg of horse spleen apoferritin were incubated in 5 ml of 6M guanidine hydrochloride at room temperature for 2 hrs prior to applying to the column to allow dissociation of the protein into its subunits. A flow

rate of 18 ml/hr was employed and fractions of 3 ml were collected. The fractions of interest were pooled separately and dialyzed against several changes of 10mM Tris pH 7.2 then lyophilized. These samples were characterized by electrophoresis in gradient-pore polyacrylamide SDS gels as described in a later section.

Amino Acid Analysis

Amino acid analysis was performed on an LKB 4010 amino acid analyzer. The amino acids were separated on an Ultropac 10 resin using the following buffer system:

0.2N sodium citrate buffer, pH 3.25 containing 2% (v/v) isopropanol
0.2N sodium citrate buffer, pH 4.25
1.2N sodium citrate buffer, pH 6.25

Samples were prepared for amino acid analysis using the following procedure:

The sample, in 6N HCl (AR grade), was placed in a hydrolysis tube (Pierce) and flushed with oxygen-free nitrogen for several minutes. The sample was de-aerated using a water pump then sealed. After incubating at 110°C for 16 hrs the hydrolysate was placed in a round bottomed flask and the hydrochloric acid removed by

rotary evaporation. The hydrolysate was washed twice with water (AR grade) and re-evaporated before finally dissolving in 0.2M sodium citrate buffer pH 2.2.

A typical automated procedure for amino acid analysis is outlined below:

Analysis:	0.2N sodium citrate buffer, pH 3.25	4 min
	0.2N sodium citrate buffer, pH 4.25	39 min
	1.2N sodium citrate buffer, pH 6.25	78 min
Regeneration:	0.4N sodium hydroxide	5 min
Equilibration:	0.2N sodium citrate buffer, pH 3.25	66 min
Temperature:	50°C for 40 minutes then at 70°C for remainder of run.	

2% (v/v) isopropanol was added to the first buffer, 0.2N sodium citrate buffer pH 3.25, to improve the separation of threonine and serine.

The quantitation of amino acids in a sample was determined against a standard containing known amounts of given amino acids. The standard hydrolysate mixture (LKB) contained 12.5nmol/ml in 0.2M sodium citrate buffer pH 2.2 of the following amino acids:

Aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, ammonia and arginine. (Cystine was present at a concentration

of 6.25nmol/ml). The standard hydrolysate mixture was analysed every 6-7 sample runs.

For manual evaluation of the results, with an accuracy of ±3%, the following procedure was followed. The base line and total height value of each amino acid peak was determined. Subtracting the baseline value from the peak height gave the net height. When the value for the net height was divided by 2 and added to the baseline value this gave the true half height of the peak. The width at this point was determined, and by multiplying this value by the net height the peak area was obtained.

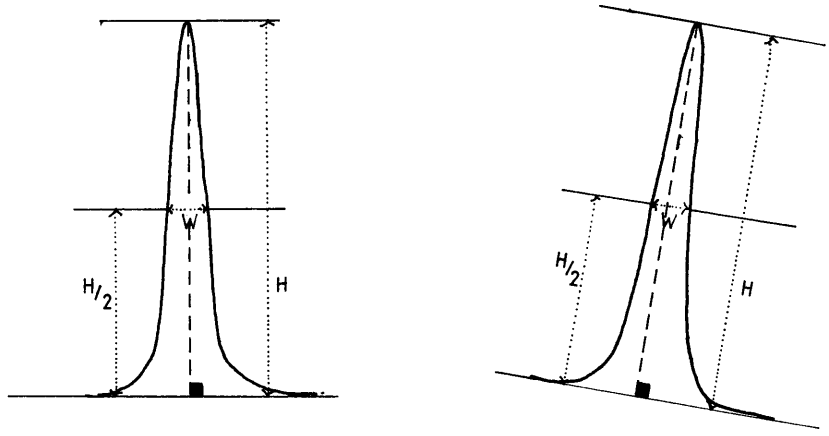


Figure 20: Procedure for manual calculation of amino acid concentration.

If the baseline was not parallel to the chart grid the peak area could still be calculated as described above except that the height should be drawn perpendicular to the sloping baseline as shown schematically above. By calculating the area (A) for a known concentration (C)

of an amino acid from the standard run, the concentration (C_X) of the same amino acid in a sample solution could be determined from its peak area (A_X) using the equation:

$$\frac{C_X}{A_X} = \frac{C}{A}$$

Electrophoresis

Sodium Dodecyl Sulphate Electrophoresis

Gradient-pore polyacrylamide gels containing sodium dodecyl sulphate (SDS) were routinely prepared using an LKB 11300 Gradient Mixer. The gels were cast between glass plates (16 x 16 cm x 1.5 mm) using the Bio-rad Protean Electrophoresis Cell. All materials used were specially purified for electrophoresis and purchased from Bio-Rad. The gels contained an acrylamide gradient of 6-22% T, 5% C and were prepared from the following solutions.

Dense solution:

17.6 ml acrylamide stock solution (50% T, 5% C)
10.0 ml 1.5M Tris buffer pH 8.8
8.0 ml water
0.4 ml 10% (w/v) SDS solution
0.25 ml ammonium persulphate solution (10 mg/ml)
20µl N,N,N',N'-tetramethylethylenediamine (TEMED)
4.0 ml glycerol

Light solution:

10.0 ml 1.5M Tris buffer pH 8.8
27.5 ml water
0.4 ml 10% (w/v) SDS solution
2.5 ml ammonium persulphate solution (10 mg/ml)
20µl TEMED

The gel was overlayed with 0.1% (w/v) SDS solution and allowed to polymerize. A stacking gel 4% T, 5% C, pH 6.8 was then cast on top of the running gel.

Stacking gel:

1.6 ml acrylamide stock solution (50% T, 5% C)

5.0 ml 0.5M Tris buffer pH 6.8

12.4 ml water

0.2 ml 10% (w/v) SDS solution

1.0 ml ammonium persulphate solution (10 mg/ml)

10µl TEMED

Samples were prepared for SDS electrophoresis by incubating at 100°C for 10 min in sample buffer.

Sample buffer:

0.125M Tris buffer, pH 6.8 containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol. Bromophenol blue was added as a tracking dye.

Electrode buffer:

30 g Tris

144 g glycine

5 g SDS

The above reagents were dissolved in distilled water and made up to 5 litres.

The gels were electrophoresed at 15mA constant current until the bromophenol blue had entered the separating gel, then electrophoresis was continued at 30mA for the remainder of the run. After electrophoresis the gels were stained with Coomassie Blue R-250 and destained by slow leaching of the dye.

Coomassie Brilliant Blue Staining Solution:

2.5 g Coomassie Brilliant Blue R-250

454 ml methanol

92 ml glacial acetic acid

The final volume of 1 litre was made up with distilled water.

Destaining solution:

750 ml glacial acetic acid

500 ml methanol

The final volume of 10 litres was made up with distilled water.

Silver Staining Procedure

When the concentration of protein applied to a gel was known to be low the gel was silver stained. This is a highly sensitive method for detecting proteins in polyacrylamide gels - up to 50 times more sensitive than

Coomassie blue R-250. A silver staining kit was purchased from Bio-rad. This consists of three reagents oxidizer, silver stain and developer and is based on the method by Merril *et al* (Merril, Goldman, Sedman and Ebert, 1981).

The protocol followed was as shown in Table 6.

Molecular Weight Estimation

Molecular weight estimations were carried out on gradient-pore polyacrylamide gels containing SDS using essentially the methods of Lambin, Rochu and Fine (1976) and Poduslo and Rodbard (1980).

In 1976 Lambin, Rochu and Fine demonstrated an empirical relationship between the logarithm of the molecular weight, log (Mr), of the protein and the logarithm of the gel concentration, log (% T) to which the protein migrated. This relationship provides a better linearity over a wide molecular weight range than a plot of log (Mr) versus the logarithm of the relative mobility, log (R_m).

Gradient-pore gels were prepared with a linear gradient of 6-22% T, 5% C, pH 8.8 and a stacking gel of 4% T, 5% C, pH 6.8, as described earlier.

Reagent	Volume	Duration (min)		
		<0.5 mm gel	0.5-1.0 mm gel	>1.0 mm gel
1. Fixative. 40%(v/v) methanol, 10%(v/v) acetic acid	400 ml	30	30	60
2. Fixative. 10%(v/v) ethanol, 5%(v/v) acetic acid	400 ml	15	15	30
3. Fixative. 10%(v/v) ethanol, 5%(v/v) acetic acid	400 ml	15	15	30
4. Oxidizer	200 ml	3	5	10
5. Deionized Water	400 ml	-	5	10
6. Deionized Water	400 ml	-	5	10
7. Deionized Water	400 ml	-	-	10
8. Silver Reagent	200 ml	15	20	30
9. Deionized Water	400 ml	-	1	2
10. Developer	200 ml	30 sec	30 sec	1
11. Developer	200 ml	~5	~5	~5
12. Developer	200 ml	-	~5	~5
13. Stop. 5% acetic acid	400 ml	5	5	5

Table 6: Protocol for silver staining polyacrylamide gels.

The following proteins were used as molecular weight markers:

Protein	Molecular Weight
Insulin Chain B	3,400
Aprotinin	6,500
Cytochrome C	12,500
Lysozyme	14,388
Myoglobin	17,200
Trypsin Inhibitor	21,500
Lactate dehydrogenase	35,000

The standard proteins were prepared at a concentration of 2 mg ml^{-1} in 0.125M Tris pH 6.8, 1% (w/v) SDS, 1% (v/v) 2-Me and incubated at 100°C for 10 min. 10µl were then applied to the gel.

After electrophoresis the gel was stained with Coomassie Brilliant Blue R-250 and destained.

The gel concentration to which the standard proteins migrated were calculated and a plot of log (% T) versus log (Mr) drawn. The molecular weights of the sample proteins could then be calculated from their log (% T) values.

Preparative SDS Electrophoresis

Preparative SDS electrophoresis was performed on an LKB 7900 Uniphor.

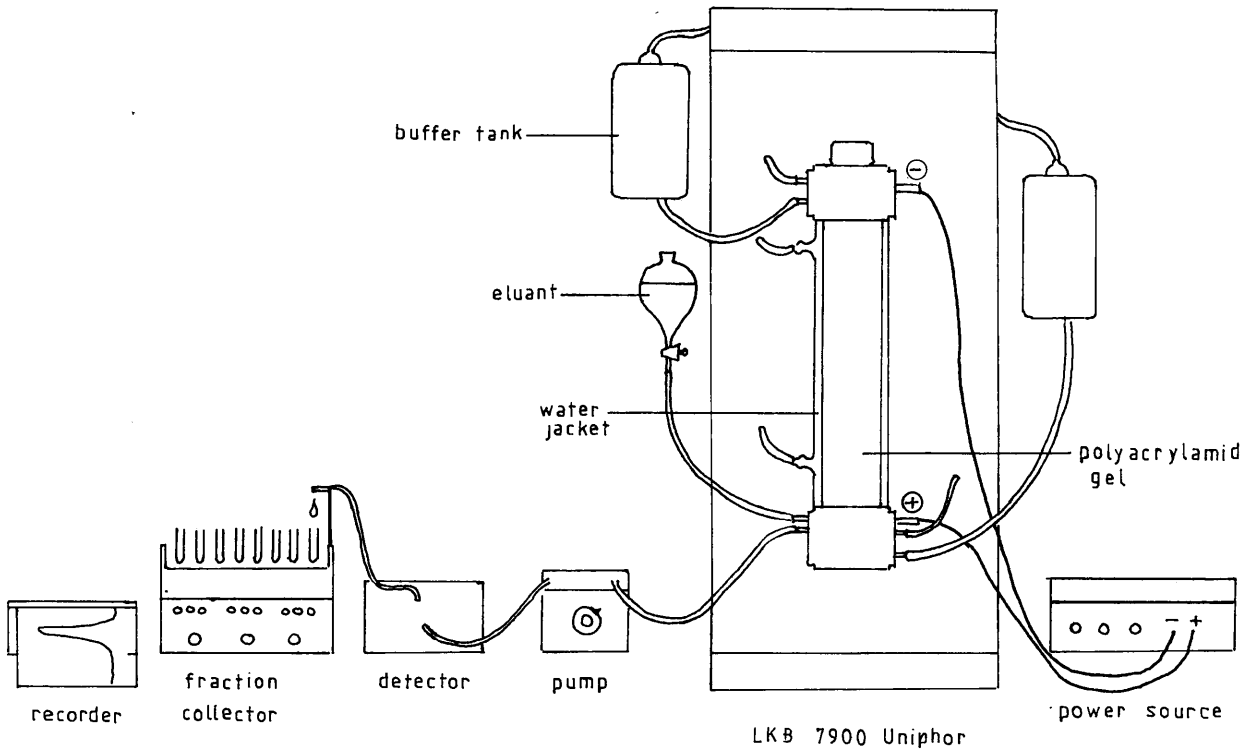


Figure 21: The LKB 7900 Uniphor

A 6-20% T, 5% C gradient gel was poured using an LKB 11300 Gradient Mixer over a period of 1 hr. Water at 12 °C was circulated through the water jacket surrounding the electrophoresis column to prevent any shrinkage of the gel caused as a consequence of the heat of polymerization.

0.1% (w/v) SDS solution was overlayed on top of the gel which was then allowed to polymerize.

Dense solution:

66 ml acrylamide stock solution (50% T, 5% C)
37.5 ml 1.5M Tris buffer, pH 8.8
31.0 ml water
1.5 ml 10% (w/v) SDS solution
0.75 ml ammonium persulphate solution (10 mg/ml)
60µl TEMED
15 ml glycerol

Light solution:

37.5 ml 1.5M Tris buffer, pH 6.8
103.0 ml water
1.5 ml 10% (w/v) SDS solution
6.0 ml ammonium persulphate solution (10 mg/ml)
60µl TEMED

Once the gel had polymerized a stacking gel 4% T, 5% C pH 6.8 was applied to the top of the separating gel.

Sample Preparation

A sample containing 25 mg horse spleen ferritin was incubated in 2 ml sample buffer at 100°C for 10 min.

It was then centrifuged for 15 min in an Eppendorf microfuge at 10,000 g to remove the insoluble iron micelles and the supernatant was applied to the surface of the stacking gel.

Electrophoresis was performed at 10W constant power. The material was eluted from the base of the gel with electrode buffer using a flow rate of 20 ml/hr. The absorbance of the eluent was monitored at 280nm using an LKB 8300 Uvicord and continuously recorded using an LKB 2210 single channel recorder.

The fractions containing protein were dialyzed extensively against 1mM Tris, pH 7.2 and lyophilized (Freeze Drier Unit - Birchover Instruments Ltd.).

Preparative Electrophoresis

Preparative electrophoresis in non-denaturing gels was used as a final stage in the purification of ferritin. A 6-22% gradient-pore gel was prepared as described previously omitting SDS from the solutions. Once the stacking gel had polymerized the gel was placed at an angle and 1% (w/v) agarose (LKB) in 0.125M Tris buffer, pH 6.8 was applied to the corners of the gel. This prevents any leakage of the sample down the sides of the gel.

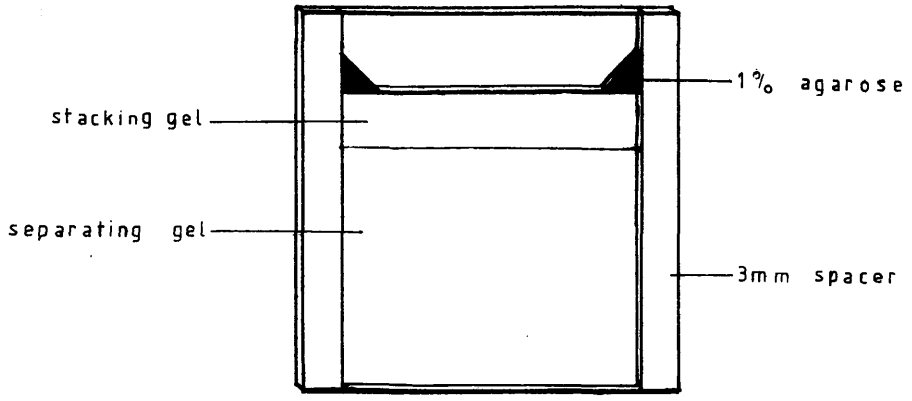


Figure 22: Apparatus for preparative electrophoresis.

The sample, containing up to 5 mg protein, was applied to the gel. Electrophoresis was performed at 20mA constant current until the tracking dye had reached the separating gel then at 40mA for the remainder of the run. After electrophoresis the brown band containing ferritin was cut out from the gel. Ferritin was recovered by passing the polyacrylamide through the needle of a hypodermic syringe and eluting into 1mM Tris buffer pH 7.2.

Two-Dimensional SDS Electrophoresis

In the present study a two-dimensional diagonal technique was devised using horizontal slab gel electrophoresis to investigate the presence of inter- and intramolecular disulphide bonds in apoferritin. A 15% T, 5% C polyacrylamide gel containing SDS was set up by

using an LKB 2117 Multiphor electrophoresis system with a plain glass plate replacing the slot former. Once the gel had set the origin was made by using a coaxial gel puncher of 2.5 mm diameter. A sample of horse spleen apoferritin was prepared for electrophoresis in the absence of any thiol reagent as described previously and electrophoresed in the first dimension for 5 hr at 7mA constant current. A 10% (v/v) solution of 2-mercaptoethanol in 50mM Tris-glycine buffer was applied to the horizontal slab gel and left to diffuse into the gel for 2 hr, after which the gel was electrophoresed at right angles to the first dimension for 8 hr at 15mA constant current. The gel was then stained with Coomassie Blue and destained as described previously.

Trans-Blot Electrophoresis

Blotting onto nitrocellulose has been a routine method of DNA transfer since Southern (Southern, 1975) developed the technique in 1975. Since that time the procedure has been extended to include the transfer of RNA and proteins to nitrocellulose and diazobenzyloxymethyl paper. Transfers were performed by capillary action until 1979 when Towbin *et al* developed a technique to transfer proteins to nitrocellulose using an electric current (Towbin, Staehelin and Gordon, 1979).

The Trans-Blot Transfer apparatus and nitrocellulose sheets (20 cm x 20 cm) were purchased from Bio-Rad.

After electrophoresis the gel was placed on a 'Scotch-Brite' pad which had previously been saturated in transfer buffer. A pre-wetted nitrocellulose paper was carefully placed on top of the gel to avoid trapping any air bubbles. A second saturated 'Scotch-Brite' pad was placed on top of the transfer paper and the gel-blot sandwich placed in the electrophoresis apparatus. The transfer paper was anodal to the gel.

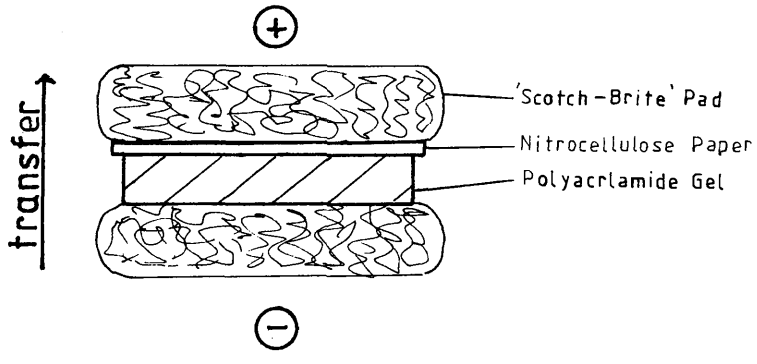


Figure 23: Diagram of electroblotting procedure.

Electrophoresis was performed at 60V for 3 hrs. The nitrocellulose paper was removed and stained with Amido Black solution then destained by leaching of the dye.

Transfer buffer

25mM Tris buffer, 192mM glycine pH 8.3 containing 20% (v/v) methanol.

Amido Black solution

2.5 g Amido Black
454 ml methanol
92 ml glacial acetic acid
454 ml water

In some experiments the nitrocellulose electroblot was used as a quick and accurate means of locating the position of the protein bands in polyacrylamide gels. In these cases electrophoresis was shortened to between 15-30 min to effect a non-quantitative transfer and the procedure used was slightly modified as outlined below.

After standard electrophoresis, the gel was placed on a wetted nitrocellulose paper then, using a solution of bovine serum albumin (1 mg/ml) a line was drawn around the edges of the polyacrylamide gel. This allowed the gel to be correctly aligned on top of the nitrocellulose paper after it had been stained. After the shortened electrophoretic transfer the electroblot was stained as before and the protein bands were visible on the nitrocellulose paper within about 5 minutes. On the basis of the electrophoretic band pattern on the electroblot,

individual protein zones could then be cut from the original polyacrylamide gel. To recover the protein the slices of polyacrylamide gel were passed through the needle of a hypodermic syringe and eluted into 1mM Tris buffer, pH 7.2.

Chemical Modification and Cleavage Procedures

Cleavage of Tryptophan Residues using o-Iodosobenzoic Acid

o-Iodosobenzoic acid is a mild and selective reagent for the cleavage of tryptophanyl peptide bonds in high yields (Mahoney, Hermondson, 1979). Cleavage occurs on the carboxyl side of the peptide bond giving rise to a C-terminal spirolactone.

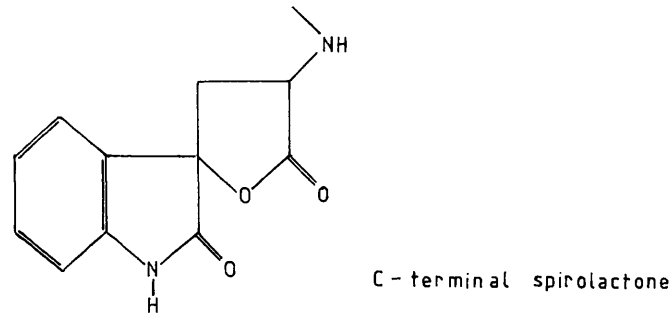


Figure 24: Cleavage at tryptophan residues using o-Iodosobenzoic acid.

Horse spleen ferritin was purified by preparative electrophoresis in a non-denaturing polyacrylamide gel as described earlier. The brown band of ferritin was cut from the gel and eluted into 50mM Tris pH 8.0. Apoferritin was prepared from ferritin by dialysing against 1% (v/v) thioglycollic acid, pH 5.5 for 24 hrs. followed by extensive dialysis against 1 mM Tris-HCl, pH 8.0. The resultant solution was clarified by centrifugation and the supernatant freeze-dried.

Apoferritin was reduced and carboxymethylated by the method of Crestfield, Moore, Stein (1963).

To 40 mg of protein were added 3.61 g deionized crystalline urea, 0.30 ml EDTA solution (50 mg/ml), 3.0 ml 1.5M Tris-buffer pH 8.6 and finally 0.1 ml 2-mercaptoethanol. The solution was made up to 7.5 ml with distilled water and deareated by flushing with nitrogen for several minutes. The mixture was allowed to stand in the dark at room temperature for 4 hrs. 0.268 g of iodoacetic acid in 1 ml 1.0M NaOH were then added and the alkylation reaction was allowed to proceed for 30 min. The protein was recovered by passing through a G-25 column (2.6 cm x 15 cm) equilibrated with 50 mM Tris buffer pH 8.0. A flow rate of 30 ml hr^{-1} was used and fractions of 2 ml were collected. The fractions of interest were pooled and lyophilized.

The cleavage procedure was essentially that of Mahoney and Hermodson, 1979.

The following reagents were incubated together in the dark for 2 hours:

3 ml 4M guanidine hydrochloride in 80% (v/v) acetic acid
60 mg o-iodosobenzoic acid (2 x weight of protein)
2.4 mg p-cresol (4.1 mg/100 mg o-iodosobenzoic acid)

o-Iodosobenzoic acid is often contaminated with o-iodoxybenzoic acid and this causes the destruction of tyrosinyl and cysteinyl residues. Pre-incubation with p-creosol reduces the content of o-iodoxybenzoic acid by

acting as a reducing agent and also a tyrosine analogue. 30 mg of the reduced and carboxymethylated protein were then added and the mixture was kept in the dark at room temperature for 24 hrs. 100µl 2-mercaptoethanol were added to quench the reaction and the mixture was extensively dialyzed against 1mM phosphate buffer pH 8.0 following which it was lyophilyzed.

Cleavage at Cysteinyl and Cystinyl Residues using 2-nitro-5-thiocyanobenzoic acid

Materials

2-nitro-5-thiocyanobenzoic acid was purchased from Eastman Kodak Company, Rochester, New York. All other materials were standard laboratory reagents available from Merck, apart from Sephadex G-25 and Sephadex G-100 which were purchased from Pharmacia.

Method

The material used in this cleavage reaction was a preparation of horse spleen apoferritin subunits which had been purified on a Sepharose CL-6B column equilibrated with 6M guanidine hydrochloride.

The lyophilized material, 20 mg, was incubated for two hours at 37°C in 4 ml of 6M guanidine hydrochloride, 0.2M Tris-acetate buffer pH 8.0, and 10mM dithiothreitol to

ensure complete reduction of any cystinyl residues. A 10-fold excess of 2-nitro-5-thiocyanobenzoic acid over total thiol present was added and the pH rapidly adjusted to pH 8.0 with NaOH. After 15 min. at room temperature the reaction mixture was acidified by the addition of an equal volume of glacial acetic acid and dialyzed exhaustively into 50% acetic acid at 4°C. A final desalting step was carried out on the modified protein by passing the material through a Sephadex G-25 column (2.6 cm x 15 cm) equilibrated with 50% acetic acid. Aliquots of the derivatized, desalted protein were removed and evaporated to dryness under a stream of nitrogen. The residue was then ready for the next stage of the procedure namely cleavage of the amino peptide bonds of S-cyanocysteinyl residues.

The residue was dissolved in 4 ml of 6M guanidine hydrochloride, 0.2M Tris buffer pH 8.0 and incubated at 37°C for 20 hrs. The products of the cleavage reaction were separated by passing the sample down a Sephadex G-100 column (1 cm x 100 cm) which had been equilibrated with 6M guanidine hydrochloride, 0.04M potassium phosphate buffer pH 7.0. A flow rate of 9 ml hr^{-1} was used and 3 ml fractions were collected. The fractions of interest were pooled together and dialyzed extensively against 0.01M potassium phosphate

buffer pH 7.0 then lyophilized. The extent of cleavage was determined by analysing the fractions obtained on SDS gradient gels.

Second Derivative Spectroscopy for Evaluating the Tyrosine to Tryptophan Ratio

Servillo *et al* (1982) have recently demonstrated that tyrosine and tryptophan residues in proteins could be quantitatively determined using a second-derivative spectrophotometric titration of the protein in the presence of 6M guanidine hydrochloride at pH 6.5. This technique was used to quantitatively estimate the ratio of tyrosine to tryptophan residues in a highly purified sample of horse spleen apoferritin and also to study the kinetics of the unfolding of the protein subunit.

Materials

N-acetyl-tyrosinamide and N-acetyl-tryptophanamide were purchased from Sigma and 8M guanidine hydrochloride (sequanal grade) was purchased from Pierce.

The concentration of the model compounds were estimated by absorption measurements using the data of Edelhoch (1967):

$\varepsilon_{280.8nm}$ = 5690 M^{-1} cm^{-1} for N-acetyl-tryptophanamide

$\varepsilon_{275.5nm}$ = 1490 M^{-1} cm^{-1} for N-acetyl-tyrosinamide

Method

The uv-spectrophotometer employed in this study was a Perkin-Elmer 550S recording spectrophotometer equipped with second derivative facilities as standard. The settings used for all studies were a scan speed of 60nm min^{-1}, a chart speed of 120nm min^{-1}, recorder ordinate range limits were set to ± 0.5 and a response setting of 3 which corresponds to a response time of 2 seconds was used throughout.

To calculate the calibration curve, 2.5 ml 8M guanidine hydrochloride were added to both the sample and reference cuvettes. The spectrophotometer was then autozeroed from 320nm to 250nm.

100µl of a known concentration of N-acetyl-tryptophanamide in 6M guanidine hydrochloride were added to the sample cell and the spectrum scanned. Successive additions of 25µl N-acetyl-tyrosinamide in 6M guanidine hydrochloride were added until 300µl in total had been added then 50µl aliquots of N-acetyl-tyrosinamide were added up to a total addition of 900µl. The value of r = a/b (see Figure 37) was calculated for each addition and plotted against the molar ratio of N-acetyl-tyrosinamide to N-acetyl-tryptophanamide. In the present study the data was analysed and graph plotted using non-linear least squares treatment on an Apple II microcomputer interfaced to a Hewlett-Packard 7225A plotter.

To determine the tyrosine/tryptophan ratio for horse spleen apoferritin, 200µl water were added to a reference cuvette containing 2.5 ml 6M guanidine hydrochloride. 200µl of an aqueous solution of horse spleen apoferritin were added to the sample cuvette containing 2.5 ml 6M guanidine hydrochloride and the spectrum was immediately scanned. A spectrum was then taken every two minutes for 10 minutes then at 10 minute intervals for up to 100 minutes. From this data both the tyrosine/tryptophan ratio could be evaluated and also the kinetics of the protein unfolding could be determined. The data was analysed and graph plotted using a least squares treatment for a first order rate equation on the DEC 2050 main frame computer equipped with GHOST graphics software.

Nitration of Tyrosine Residues using Tetranitromethane

Spectrophotometric Determination

The method employed for the nitration of tyrosine was essentially that of Sokolovsky, Riordan and Vallee (1966).

A 1 mg/ml solution of horse spleen apoferritin in 0.05M Tris buffer pH 8.0 was prepared. A stock solution of 8.4M tetranitromethane (Aldrich) was diluted 1 in 10 with 95% ethanol.

1 ml of buffer was placed in the reference cell of a Perkin-Elmer spectrophotometer and 1 ml of the protein solution was placed in the sample cuvette. 10µl of the diluted tetranitromethane solution were added quickly to each cuvette and the absorbance at 428nm continuously recorded. After 3 hr the modified protein was desalted using a Sephadex G-25 column equilibrated with 0.01M Tris, pH 8.0 and the pooled fractions were lyophilized. The experiment was repeated within a range of temperatures between 10°C - 60°C.

Amino Acid Analysis

The extent to which the tyrosyl residues had been modified was also monitored by amino acid analysis. A sample of the desalted, modified material was hydrolysed and prepared for amino acid analysis as described previously. Using a standard of 3-nitrotyrosine the elution time of this derivative was determined using the conventional amino acid programme. This modified amino acid was found to elute after phenylalanine and before histidine.

The colour value of this amino acid was determined after it had been reacted with ninhydrin. Using a known molarity of 3-nitrotyrosine the colour constant was calculated according to Beer's Law. Thus,

$$\text{colour constant} = \frac{\text{Absorbance}}{\text{moles of amino acid}}$$

The absorbance of an unknown quantity of the amino acid could be easily converted into moles of amino acid by dividing the absorbance of the unknown solution by the colour constant.

Sequencing Studies

N-terminal Analysis using Dansyl Chloride

Method

The method used was essentially that of Gray (1972). A sample of protein (50µg - 250µg) which had been carboxymethylated or treated with performic acid was placed in a micro-test tube. 50µl of 1% (w/v) SDS were added, mixed and heated at 100°C for 5 min to ensure unfolding of the peptide chain. 50µl of N-ethylmorpholine were then added and mixed. A final addition of 75µl of a solution of dansyl chloride (25 mg ml^{-1} in N,N dimethylformamide) was made and the resulting reaction mixture left for 1 hour at room temperature.

In order to precipitate the labelled protein 0.5 ml acetone was added to the reaction mixture. The protein usually precipitated as fine floccules which were formed into a pellet by centrifuging for a few minutes in a bench centrifuge. The supernatant was removed and the pellet of protein was washed again with 0.5 ml 80% (v/v) acetone then re-centrifuged and dried ready for hydrolysis.

Before adding the hydrochloric acid, the tube was pinched with a gas/oxygen flame. 50µl of 6M HCl were then added and the sample centrifuged for approximately

1 minute. The mixture was deaerated very gently and while still deaerating the neck of the flask was sealed with a gentle gas flame. The tube was placed in an oven at 110°C for 4 hours. At the end of the hydrolysis period the tube was opened and the hydrochloric acid removed *in vacuo* over NaOH pellets.

Identification of dansyl amino acids

The DNS-amino acids were identified by thin layer chromatography based on a 2-dimensional procedure developed by Woods and Wang (1967). Micropolyamide sheets were used which were coated on both sides. These sheets were purchased from Schleicher and Schüll (356000) and normally cut to 5 cm x 5 cm. In order to identify all the DNS-amino acids three or four solvent systems are used with a re-run in the second dimension using the third and fourth solvents.

The DNS-amino acid was dissolved in 10µl of 95% (v/v) ethanol. A small aliquot (1 - 5µl) of a standard solution of DNS-amino acids was applied to one corner of the polyamide sheet. In the same corner, but on the other side of the sheet the sample was applied. The sheet was then placed in a small tank which had been equilibrated with the first solvent. After the solvent front had almost reached the top of the sheet it was removed from the tank, dried, turned through 90° and

re-run in the second solvent. At this stage the sheet was examined under ultra-violet light to locate the fluorescent DNS-amino acids and the DNS-amino acids were identified where possible. At this point particular attention was paid to DNS-leu/DNS-ile since this separation may be obscured at a later stage. DNS-thr/DNS-ser and DNS-asp/DNS-glu partly overlap and may be resolved by an additional run with a third solvent in the same direction as solvent 2.

Solvent 1: Formic acid: Water, 1.5 : 98.5 (v/v)
Solvent 2: Toluene: Acetic acid, 10 : 1 (v/v)
Solvent 3: Ethanol: Methanol : Acetic Acid, 20 : 1 : 1 (v/v/v)

After each dimension the polyamide sheet was dried to remove all traces of solvent before running in the next dimension.

In some cases a fourth solvent was used in order to resolve α-DNS-lys/DNS-arg and ε-DNS-lys.

4th Solvent: 0.05M Na_3PO_4 : ethanol, 3 : 1 (v/v)

Determination of the amino acid sequence of proteins and peptides were carried out using an LKB 4030 solid phase sequencer. In the solid-phase method of protein sequencing the protein or peptide to be sequenced is attached to an inert support through either the C-terminal carboxyl group or through a side chain function. The sequence of reactions then follows the Edman degradation procedure.

Coupling Procedure

With large peptides and proteins it is preferable to activate the porous glass support using p-phenylenediisothiocyanate (DITC) before adding the peptide rather than adding activated peptide to the glass support. Using this method an increase in coupling yield is obtained.

The following procedure was followed:

Aminopropyl glass was activated by adding 200 mg of 3-aminopropyl glass in small aliquots to a solution of DITC in dimethylformamide (DMF). 2-3 volumes DMF per volume of glass were used containing a 50 molar excess of DITC over amino groups. After stirring briefly the mixture was allowed to stand for 2 hours at room temperature. The derivatized glass was then washed with DMF then methanol and dried in a vacuum. Alternatively 200 mg commercially prepared activated glass was used.

200nmol of protein or peptide was rendered ammonia free by drying down twice from 250µl of 5% (v/v) aqueous triethylamine. 200 mg of activated glass was suspended in 0.4 ml 50% (v/v) aqueous pyridine, 0.4M dimethylallylamine-trifluoroacetic acid pH 9.0 (DMAA)(Pierce) and a solution of the peptide in the same solvent (0.4 ml) was added. The mixture was degassed under a water pump, then gently swirled under oxygen-free nitrogen for 1 hour at 40° C. To ensure complete phenythiocarbamylation of the α-NH_2 group 20µl phenylisothiocyanate were added and incubated under nitrogen for a further 30 min at 40° C. The beads, with the attached peptide were then washed on a sintered glass funnel with 2 ml of DMAA solvent and resuspended in 0.4 ml of the same solvent. Remaining isothiocyanate groups on the glass support which could interfere with the subsequent degradation were blocked by the addition of 100µl ethanolamine and incubated under oxygen-free nitrogen at 40°C for 30 min. The glass was then collected on a filter funnel washed with 2 ml DMAA buffer and then with 4 ml methanol and dried. Trifluoroacetic acid (0.4 ml) was added and the mixture incubated under nitrogen at 40°C for 20 minutes. The glass was collected by filtration, washed with methanol and dried. Edman degradation was then performed from the second residue.

Determination of the Coupling Yield

To determine the amount of peptide which had attached to the support, an aliquot of the support material was taken and subjected to acid hydrolysis.

5 mg of support were washed with TFA, to remove any unbound peptide, then transferred to a hydrolysis tube. 2 ml of 6M HCl were added and the sample hydrolyzed at 110°C for 16 hr. Amino acid analysis of the hydrolysate indicated the number of nanomoles of sample bound to the support material.

Reagents and Solvents

All reagents and solvent were sequanater grade and purchased from LKB Cambridge.

TFA: Anhydrous trifluoroacetic acid (100 ml)

PITC: 5% (v/v) phenylisothiocyanate in acetonitrile (100 ml)

Buffer: 5.8 ml N-methylmorpholine mixed with 34 ml distilled deionized water and adjusted to pH 8.4 with TFA (~0.5 ml)
60 ml pyridine was then added (100 ml)

DCE: Dichloroethane (500 ml)

MeOH: Methanol (500 ml)

Sequencing

The LKB 4030 Solid Phase Sequencer consists of three main sections a control unit, the fluidics system and

the column and fraction collector as shown below

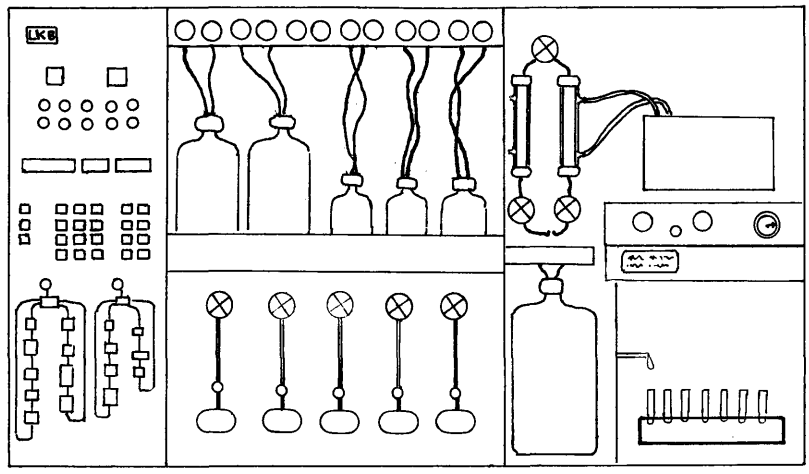
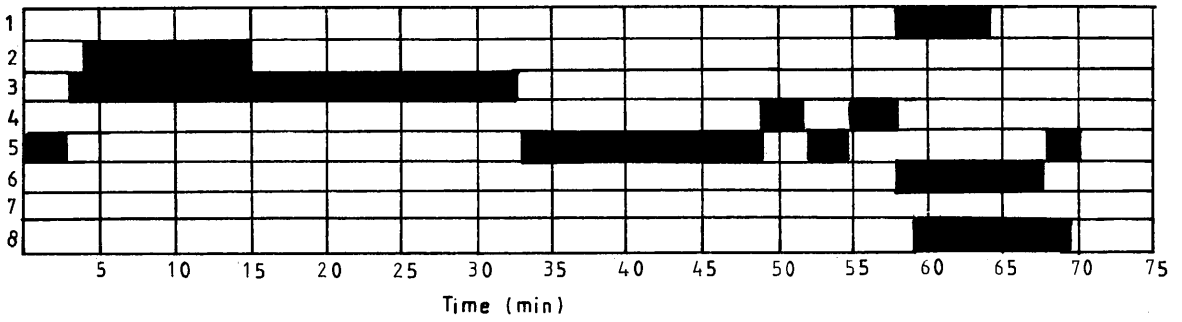


Figure 25: The LKB 4030 Solid Phase Sequencer

The left hand section of the instrument contains an electronic control panel. This unit allowed the sequencer to be operated manually or automatically by selecting a programme from the programme library. Programmes were available for single and dual column operations and microsequencing using ^{35}S-PITC. The machine had the facility to edit programmes to suit the particular requirements of the material to be sequenced e.g. changing the time at which certain reagents were allowed to pump through the column or altering the overall length of a sequence cycle. The number of cycles to be sequenced was selected by the user before the start of sequencing after this the machine operated automatically

until the specified number of cycles had been completed.

A typical programme for a single column operation is shown below:



1 TFA pump
2 PITC pump
3 Buffer pump
4 DCE pump
5 MeOH pump
6 Column 2 select
7 Column 2 collect
8 Column 1 collect

Figure 26: A typical single column sequencing programme

The central section of the instrument houses the solvent and reagent reservoirs, pumps and valves necessary for sequencing. Edman Degradation was automatically performed by pumping the necessary reagents through the reaction column in a specified sequence which was determined by the programme selected. Any excess reagents and by-products were washed from the column by the solvents methanol and 1,2-dichloroethane and went to waste. Towards the end of a sequence cycle TFA was pumped onto

the column to release the PTH amino acid derivative. At this point the eluent was diverted from waste to a fraction collector and any remaining PTH amino acid was washed from the column with methanol and collected. Two minutes were added to the end of each cycle to allow the pumps to refill before the next cycle commenced.

All reagents and solvents were maintained under a nitrogen atmosphere.

The right-hand section of the instrument contains the reaction column, associated valves, water bath, fraction collector, waste reservoir and nitrogen controls.

The reaction columns were maintained at 50°C by circulating water through their surrounding water jackets from a thermostated water bath. The fraction collector was able to collect individually the eluent from either one or both reaction columns for up to 24 sequence cycles.

Conversion of Anilinothiazolinone to Phenylthiohydantoin Derivatives

Using the above sequencing strategy the amino acid is released from the reaction column as an unstable anilinothiazolinone which must be converted to the stable phenylthiohydantoin before identification.

The following procedure was followed:

The numbered test-tubes containing the anilinothiazolinone amino acids were dried under a stream of nitrogen at 50°C. 200µl 1M-HCl were added to each tube, vortexed and incubated at 80°C for 10 min. 750µl ethyl acetate were then added to each tube which was then vortexed and centrifuged at 2000 rpm for 2-3 min to separate the two phases. The upper organic layer was transferred to a numbered conical test-tube (Reacti-Vial, Pierce). The ethyl acetate extraction was repeated and the organic phases combined to give a total volume of 1.5 ml ethyl acetate. This was then dried under nitrogen at 50°C as before.

Using this procedure the PTH derivatives of histidine, arginine and cysteic acid all remain in the aqueous phase while the remaining PTH amino acids are extracted into the organic phase.

Identification of PTH-amino acids

Several methods are available for the identification of PTH-amino acids including thin-layer (tlc) high-performance liquid chromatography (hplc) and back hydrolysis to the parent amino acid followed by amino acid analysis. In general tlc was used as the first method of identification followed by one of the other methods

as confirmation of the result.

Thin layer chromatography

20 x 20 cm aluminium backed silica plates containing an internal fluorescent indicator (Merck 5554) were used for the identification of the organic phase PTH-amino acids. The PTH-amino acid was re-dissolved in 20µl ethyl acetate and 10µl (containing 5-10nmoles) were spotted onto the plate using a micro-capillary tube. 1µl of a marker mixture (LKB) was spotted at regular intervals along the origin of the chromatogram. The PTH-amino acid marker mixture contained 5-10nmoles/µl of PTH-pro, leu, ile, val, phe, ala, met, trp, gly, lys(εPTC, αPTH derivative), tyr, ser, thr, gln, asn, glu, and asp dissolved in ethyl acetate : ethanol.

Development of the Chromatogram

Two solvent systems were used:

Solvent 1: Chloroform : Ethanol 98 : 2 (v/v)
This solvent enabled the following PTH-amino acids to be identified (in decreasing order of Rf values) PTH-pro, leu, ile, val, phe, met, ala, gly, trp, lys, tyr.

Solvent 2: Chloroform : Ethanol : Methanol 88.2 : 1.8 : 10 (v/v)
This solvent enabled the remaining PTH-amino acids (in decreasing Rf values) PTH-thr, ser, gln, asn, glu and asp.

The PTH amino acids were visualized by viewing under a short-wavelength ultra violet lamp at 254nm. The PTH amino acids quenched the fluorescence of the internal indicator contained in the silica plates so that they appeared as black spots against a yellow background. The plates were photographed in the dark with an ultra-violet lamp at 254nm held over the plates. An orange filter was used in the photographic process.

Back-hydrolysis using Hydriodic Acid (HI)

After conversion from the anilinothiazolinone to the phenylthiohydantoin, the PTH derivatives of histidine, arginine and cysteic acid remain in the aqueous phase. The most reliable method for their identification was to hydrolyse them back to the parent amino acid followed by amino acid analysis.

The aqueous phase was transferred to a hydrolysis tube and dried under vacuum. 200µl of 55% (v/v) HI were added to the tube which was then flushed with nitrogen, deaerated and sealed. The tubes were incubated at 130°C for 20 hrs then opened and dried in a vacuum

dessicator over fresh NaOH pellets. The samples were re-dissolved in 1.5 ml analyzer loading buffer (0.2M sodium citrate buffer pH 2.2) and centrifuged at 9000 g for 15 min to sediment any precipitated iodine before analyzing on an LKB amino acid analyzer as described earlier.

High Performance Liquid Chromatography

Hplc was used to confirm the identity of the PTH amino acids detected by tlc.

The remainder of the organic phase PTH amino acid was dried down under nitrogen and redissolved in 15µl of the mobile phase solvent (see below). In some cases the PTH amino acids from the tlc plates were scraped off the silica plates and extracted with ethyl acetate before being dried under nitrogen and re-dissolved in the mobile phase. 10µl samples were applied to a Spherisorb 5 ODS column (Chrompack). The dimensions of this column were as follows: length 25 cm, internal diameter 4.6 mm, external diameter $\frac{1}{4}$ inch. An Altex pump was used to maintain a flow rate of 1 ml min^{-1} and a pressure of 70 bar throughout the run. The column eluant was monitored at 254nm using a Cecil CE 2012 reference channel variable wavelength uv monitor which was connected to a Hewlet Packard 3390A integrator. This allowed the R_f values and peak areas of the PTH amino acids to be determined.

Mobile phase:

The mobile phase was prepared from hplc grade reagents purchased from Fisons.

34% (Tetrahydrofuran : acetonitrile : methanol, 70 : 20 : 10 (v/v))

66% (0.015M sodium acetate buffer pH 4.6)

Using individual PTH amino acids (Fluka), the R_f values, and peak areas, of these standard amino acids could be determined. When a sample was injected onto the column the PTH amino acids could be identified by their Rf values and the main PTH amino acid could be distinguished from any background or overlapping PTH amino acids by the peak areas. Background PTH amino acids arise as a result of random hydrolysis of the peptide, this is especially a problem with larger peptides and proteins. Overlapping PTH amino acids occur to some degree when the degradation is out of step from one cycle to another.

Cell Culture

Studies on the Biosynthesis of Ferritin in Morris Hepatoma Cells

A study involving cell culture experiments was undertaken while on a two month travel fellowship awarded by the Albert Einstein College of Medicine in New York, U.S.A. This award was provided for a research programme in collaboration with Professor Irvine Listowsky to work on specific aspects of the biochemistry of a characterised Morris hepatoma cell line.

Cell Culture

The Morris hepatoma cell line, $H_4A_Z C_2$, used in these studies was kindly donated by Dr. L. Reid (Albert Einstein College of Medicine). The cells were cultured in 75 ml disposable tissue culture flasks (Corning) in RPMI 1640 media (Gibco) containing 10% foetal calf serum (Gibco). The flasks were incubated at 37°C under 5% CO_2 in air. In order to maintain the cell line the cells were sub-cultured every three days.

Iron Overloading Studies

Morris hepatoma cells were seeded (2.6×10^5 cells/dish) onto 60 mm petri dishes (Falcon) in RPMI 1640 media

containing 10% foetal calf serum. One day after seeding, the media was removed and replaced with iron-supplemented media. The iron donors used in these experiments were ferric ammonium citrate (FAC) and ferric nitrilotriacetate (FeNTA) at concentrations of 50μM, 100μM and 200μM. These were prepared as described by Jacobs *et al* (1978). The cells were harvested daily after removing the media and washing once with 20mM phosphate buffered saline, pH 7.4 (PBS). The cells were dispersed in the same buffer containing 2mM phenylmethylsulphonyl fluoride (PMSF) and counted, in triplicate, using a haemocytometer.

After counting, the cells were pelleted by centrifuging at 2,000 g for 10 minutes. The supernatant was discarded and the cells resuspended in PBS containing 20mM PMSF, 0.02% NaN_3. In order to disrupt the cell membranes this cell suspension was sonicated for 3 x 10 seconds. The resulting solution was heated to 70°C for 10 minutes, to precipitate cellular proteins other than ferritin, followed by centrifugation at 20000 g for 30 minutes. The supernatant was recovered to be used in both 2-site and competitive radioimmunoassays.

Preparation of rabbit anti-ferritin serum

The preparation of rabbit antisera against rat liver ferritin was based on the method of Marcus and Zinberg (1974), using purified rat liver ferritin (as prepared on page 65) as the antigen. Anti-rat liver ferritin serum thus obtained did not give crossed immunoprecipitation lines when examined by the Ouchterlony double

diffusion method indicating that no cross-reaction occured with other cellular components. The IgG fraction was obtained from the antisera by precipitation with sodium sulphate then applied to a diethylaminoethyl cellulose (DEAE)-column (15 x 1.0 cm) (Pharmacia).

A further purification step was sometimes carried out by applying the IgG fraction to an affinity column of CNBr activated Sepharose (Pharmacia) to which rat liver ferritin had been previously immobilized. After washing the column with phosphate buffered saline the anti-ferritin antibody was eluted from the column with 3M potassium thiocyanate and dialysed against 20mM phosphate buffered saline overnight at 4°C.

The lactoperoxidase method was used to radioiodinate purified anti-rat liver ferritin antibodies. Enzymobeads were purchased from BioRad as they provide mild reaction conditions for radiolabelling the purified antibodies with ^{125}I. The procedure used was as described in the manufacturers instructions:-

The Enzymobeads were rehydrated with 0.5 ml distilled water at 4°C for at least 1 hr before use. A solution of 1% (w/v) D-L-glucose was prepared. The following reagents were added to a disposable test tube:

0.2M phosphate buffer, pH 7.2	50µl
antibody sample in azide free buffer	10-25µl
Enzymobead reagent	50µl
$Na^{125}I$	5-25µl
1% glucose solution	25µl

The reagents were mixed and the radioiodination allowed to proceed for 15-25 minutes at room temperature. To quench the reaction, the contents of the test tube were applied directly to a Sephadex G-25 column (1.0 cm x 15 cm) equilibrated with 20mM sodium phosphate buffer, pH 7.4 containing 1% (w/v) bovine serum albumin. 0.5 ml fractions were collected and their radioactivity measured in a Packard gamma counter. The specific activity of the radiolabelled antibody was 6.5µCi/µg protein.

2-site Radioimmunoassay

Polyvinyl chloride flexible microtiter plates (Dynatech Laboratories, Inc.) were employed as the solid phase. The coating procedure was based on the method described by Watanabe *et al* (1979). 200µl of the IgG fraction (60µg/ml) in buffer A was dispensed into each well of the plate and kept at room temperature for 30 minutes then at 4°C overnight. The microtiter plates were washed three times with buffer A. Each well of the IgG coated

plates was filled with 100µl of buffer B, then 50µl of cell extract appropriately diluted with the same buffer (or standard rat liver ferritin) were added. The plates were sealed with tape and agitated gently on a rotatory shaker at 37°C for 4 hours. After the first incubation the wells were rinsed three times with buffer B. Radiolabelled purified anti-rat liver ferritin antibody solution was dispensed into each well (200µl). The second incubation was carried out at room temperature for 16 hours. The wells were then washed four times with saline before counting the radioactivity of each well individually in a Packard gamma counter. The working range of the assay was between 15 ng to 1000 ng/ml, and the assay was performed in duplicate.

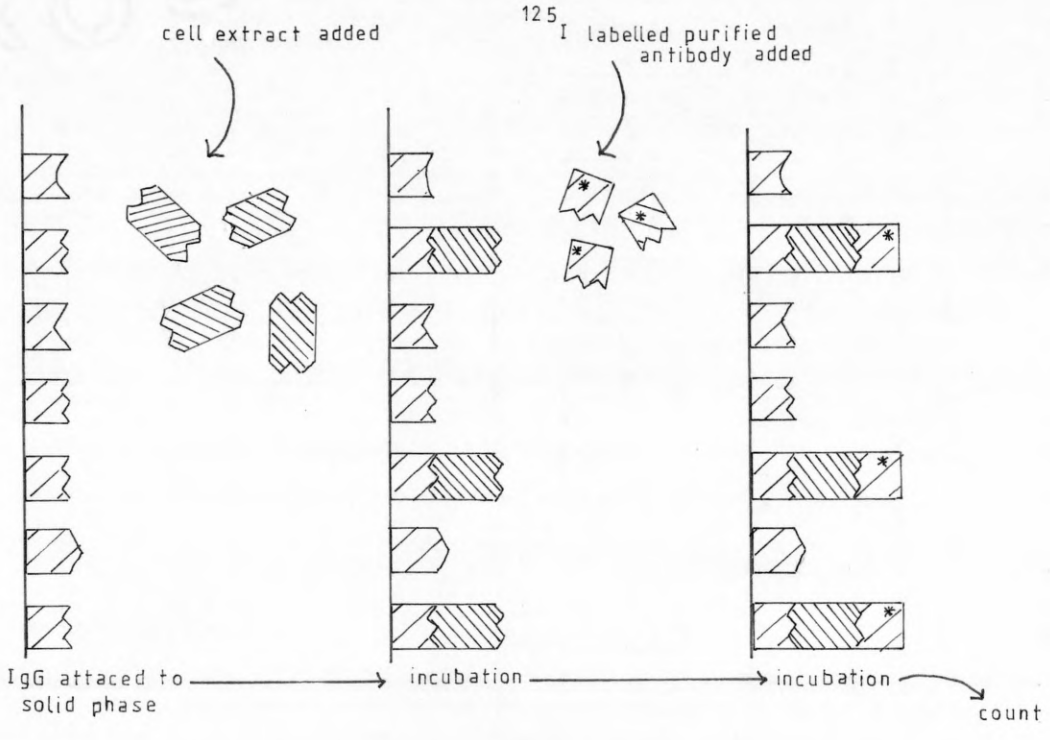


Figure 27: 2-site Radioimmunoassay

Buffer A: 0.01M sodium phosphate buffer, pH 7.0 containing 0.1M NaCl, 1mM $MgCl_2$, 1g NaN_3 and 1 g bovine serum albumin per litre

Buffer B: 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 containing 20 mg NaN_3 and 1 ml normal rabbit serum (GIBCO) per litre.

Competitive Assay

IgG coated microtiter plates were prepared as described in the two-site assay procedure. After washing the wells three times in buffer A the wells were incubated with 200µl of this buffer at 4°C overnight in order to prevent non-specific binding. This buffer was removed and the wells washed three times with buffer B. Into each well 100µl of buffer B, 50µl of cell extract appropriately diluted with the same buffer (or standard rat liver ferritin) and finally 50µl of radioiodinated rat liver ferritin were added. Rat liver ferritin was labelled with ^{125}I using the same method as described previously for radioiodination of antibodies. The plate was incubated at room temperature for 30 minutes then at 4°C overnight, after sealing the wells with tape. After washing the wells three times with normal saline each well was counted individually in a Packard gamma counter.

The working range of the assay was between 500-2000 ng, and performed in duplicate.

Study of ^{14}C-leucine uptake by hepatoma cells

Morris hepatoma cells were seeded (1 x 10^6 cells/dish) onto 30 mm petri dishes (Falcon) in RPMI 1640 media containing foetal calf serum. The cells were incubated in 5% CO_2 in air at 37°C until they had reached a confluent stage of growth. After removal of the media it was replaced with 2 ml iron conditioned media. Both ferric ammonium citrate and ferric nitrilotriacetate were used at concentrations of 25μM, 50μM, 100μM and 200μM in RPMI 1640 containing 10% foetal calf serum. 4μCi of ^{14}C-(DL) leucine (Amersham, specific activity 54 mCi/mmol) were added to each dish. After a 24 hour incubation period this media was removed and washed three times with 20mM phosphate buffered saline. The cells were lysed by the addition 2 ml phosphate buffer saline containing 0.5% Triton X-100 (Sigma) and 2mM PMSF incubated at 4°C overnight. This procedure both detaches the cells from the dishes and also disrupts the cell membranes. The lysates were centrifuged at 20,000 g for 30 min and the supernatant collected for further study.

Immunoprecipitation and gel electrophoresis

The newly synthesised ferritins in the cell lysates which had been stimulated by iron were studied by a direct immunoprecipitation assay using anti rat-liver ferritin antiserum raised in rabbit. Purified rat liver ferritin (50µg), which acted as a carrier, and anti-rat liver ferritin antibody (60µl) were added to the cell lysates. The mixture was incubated at 37°C for 30 minutes before adding an excess of goat anti-rabbit IgG antiserum (100µl) (Miles-Yeta, Ltd.) to promote co-precipitation. The complex was incubated at 37°C for 30 minutes then at 4°C overnight. Immunoprecipitates which formed during this period were centrifuged in an Eppendorf microcentrifuge and washed three times with 20mM phosphate buffered saline. The pellets were solubilized in SDS sample buffer (see electrophoresis section) and incubated at 100°C for 10 min before applying on an 8-22% T, 5% C polyacrylamide gradient-pore SDS gel. After electrophoresis, radioactivity was visualized by fluorography. Fluorography was used in preference to autoradiography because of its greater sensitivity. The gel was fixed and stained with 50% (w/v) trichloroacetic acid and 0.006% (w/v) Coomassie Brilliant Blue R-250 for 4 hours, then destained with 10% (v/v) methanol, (10% v/v) acetic acid in water. The destained gel was impregnated with EN^{3}HANCE solution (New

England Nuclear) for 1 hr with gentle aggitation as suggested by the manufacturer. Following impregnation the gel was washed and soaked in cold distilled water for 1 hr before drying in a slab gel drier (BIO-RAD).

X-ray film (5B-5 Kodak) was preflashed (0.15 A_{540} units) because only after a pre-exposure procedure does the absorbance of the fluorographic image become proportional to the sample radioactivity. The X-ray film was placed in contact with the gel and exposed at -70°C for 20 days, developed and fixed with reagents supplied by Kodak. The relative amount of the exposed bands were quantitated using by densiometric scanning at 540 nm.

RESULTS AND DISCUSSION

Electrophoresis

Structural studies on the iron storage protein, apoferritin have shown that it consists of 24 subunits arranged symmetrically around an iron-containing core. It was originally thought that the subunits were identical, however recent evidence using both isoelectric focusing and gradient-pore polyacrylamide SDS electrophoresis suggests that the observed microheterogeneity may be a result of two different types of subunit.

When ferritin from a single organ is subjected to isoelectrofocusing it reveals multiple bands, these individual bands being referred to as isoferritins. In addition, gradient-pore polyacrylamide gel SDS electrophoresis shows the presence of two subunit bands, a heavy band H, and a lighter band L with molecular weights of 21,000 and 19,000 respectively. Additional smaller molecular weight bands are also present. The isoferritins observed on isoelectrofocusing are thought to arise from combining different proportions of the H and L subunits to give a 24-mer. The explanation of this microheterogeneity represents a controversial area in ferritin biochemistry. It has been argued that the multiple bands are artifacts due to interactions between the protein and ampholytes and also may be the result of some subunits being glycosylated.

In the present research programme a number of different separation and analytical techniques have been used in order to investigate and isolate the various bands seen on polyacrylamide gels and determine their origin. Some of the factors which affect the electrophoretic profile of horse spleen apoferritin and also some human ferritins have been investigated. These are:-

i) the effect of thiol concentration
ii) peptides arising from a specific proteolytic cleavage
iii) the glycosylation of subunits

i) The effect of thiol concentration

It has been reported by several groups of workers that when horse spleen apoferritin is separated by polyacrylamide gel electrophoresis in the presence of SDS several discrete bands are visible. In addition to the subunit band(s) other bands are present which have molecular weights above and below that of the subunit. The smaller molecular weight bands are not the result of random cleavage of the protein as they are consistently found as discrete bands on PAGE gels (see later section). The bands which appear to have higher molecular weights than that of the subunit are greatly affected by the concentration of thiol in the sample buffer.

In this study the effect of thiol concentration on the band pattern obtained on SDS polyacrylamide gel electrophoresis was considered. The study also incorporated a 2-dimensional diagonal technique to identify the origin and fate of certain electrophoretic bands.

A number of different gradient-pore polyacrylamide SDS gels were investigated using both step-wise and continuous gradients. The system which appeared to give the best resolution of the protein bands was a continuous acrylamide gradient of 6-22% T, 5% C, pH 8.8 with a stacking gel of 4% T, 5% C, pH 6.8. The gels were prepared using a Tris-glycine buffer containing 0.1% (w/v) SDS. Samples were prepared for electrophoresis at a variety of thiol concentrations ranging from 0-10% (v/v) 2-mercaptoethanol in Tris buffer, pH 6.8.

When subjected to a polyacrylamide gel of this type horse spleen apoferritin resolves into a complex electrophoretic pattern. In the absence of thiol two major bands can be identified with molecular weights of 19,000 (representing the subunit) and 15,000. In addition there were minor stained bands corresponding to peptide fragments and subunit dimers. The effect of thiol (2-mercaptoethanol and dithiothreitol) on the electrophoretic pattern can clearly be seen in Figure 28 . On increasing the concentration of 2-mercaptoethanol it can be clearly demonstrated that the band of molecular weight 15,000

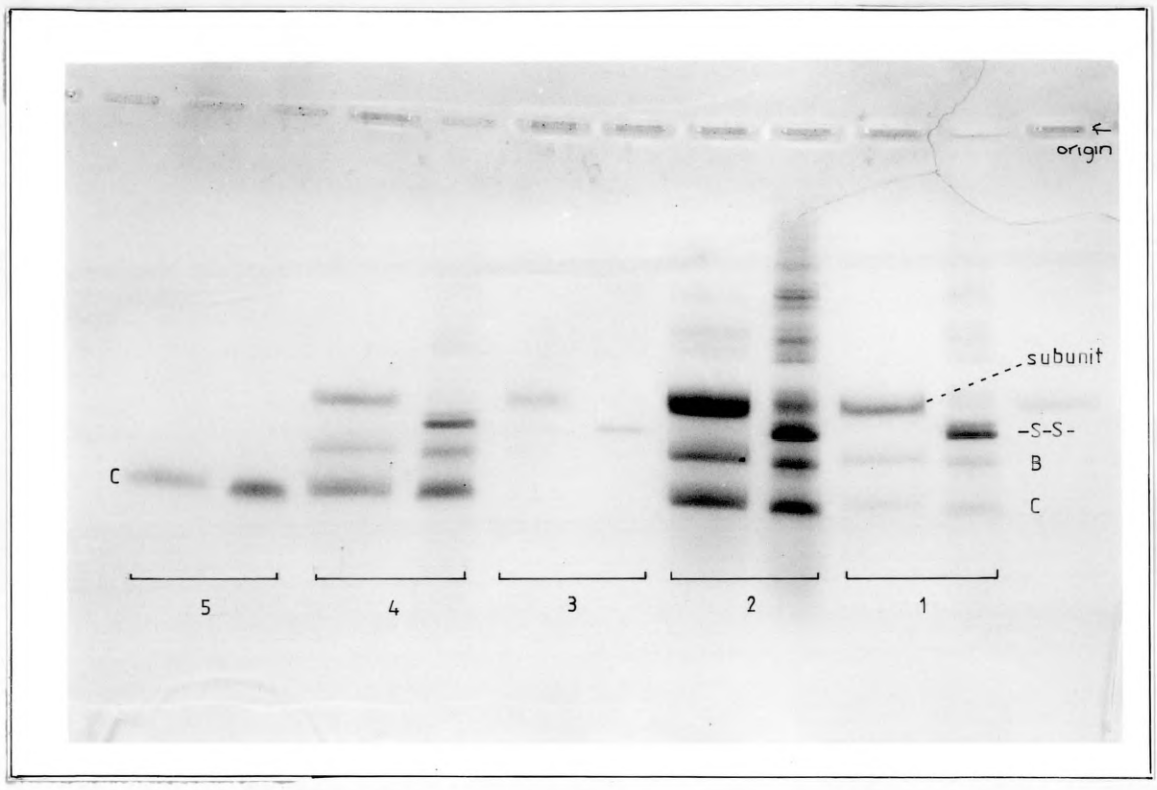


Figure 28: The effect of 2-mercaptoethanol on the elecrophoretic profile of horse spleen apoferritin.

Tracks 1-4: Samples of horse spleen apoferritin taken from a Sephacryl S-200 superfine column equilibrated with 6M guanidine hydrochloride. (See figure 30, page 129 for futher details).
5: Peptide C, also obtained from a Sephacryl S-200 column.

The samples were electrophoresed on a SDS polyacrylamide gradient gel 6-25%T, 5%C, pH 8·8. In each case the left hand track contained additionally 1% 2-mercaptoethanol.

decreases in amount and a reciprocal increase in the amount of the 19,000 molecular weight species is apparent. In addition the bands whose molecular weights appeared to be greater than 19,000 decreased when mercaptoethanol was present. These results are consistent with the interpretation that this 15,000 molecular weight material represents the subunit with an intact disulphide bond, so reducing the apparent molecular size in the presence of the denaturant, SDS. Dimers and trimers of the subunit may also be associated via disulphide linkages.

To confirm these interpretations a two-dimensional diagonal technique was devised. Using a horizontal slab gel apparatus, polyacrylamide SDS gels were prepared using a uniform concentration of acrylamide (15% T, 5% C). A sample of horse spleen apoferritin was prepared for electrophoresis in the absence of any thiol reagent. This was applied to one corner of the gel which was then electrophoresed in the first dimension. A solution of 10% (v/v) 2-mercaptoethanol was overlayed onto the horizontal slab gel and left to diffuse into the gel for two hours after which the gel was electrophoresed at right angles to the first dimension. The results of this experiment are shown in Figure 29.

From this data it is evidence that i) subunit dimers containing an intermolecular disulphide bond can exist as witnessed by the dimer into subunit conversion on

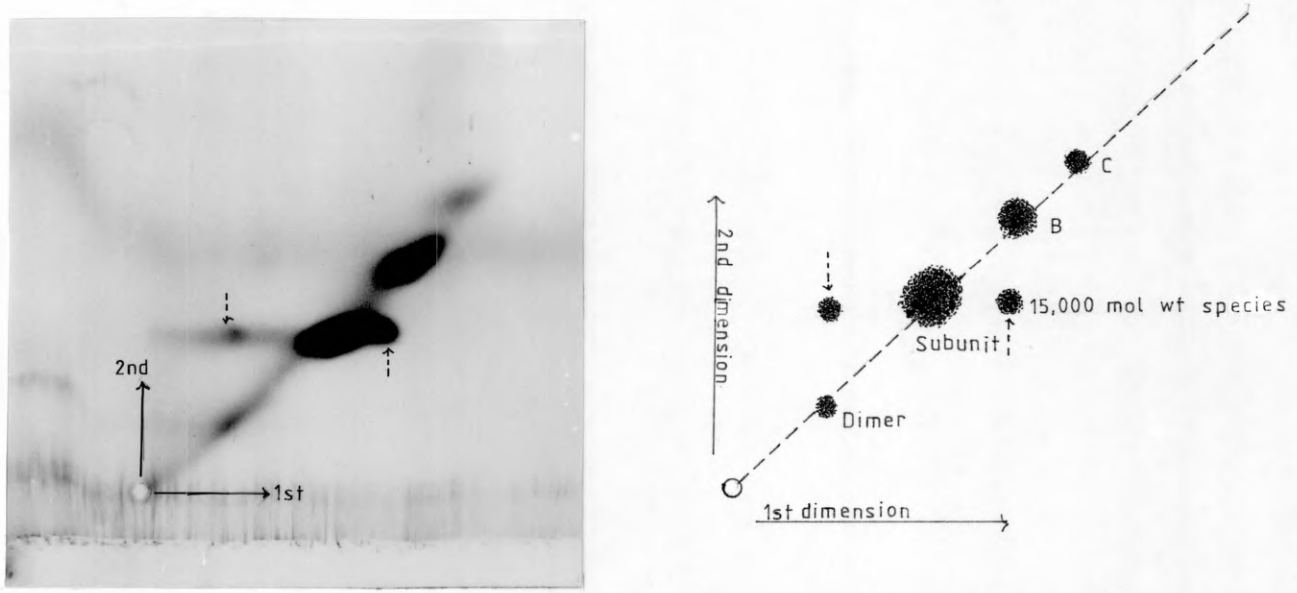


Figure 29: Two-dimensional sodium dodecyl sulphate/polyacrylamide gel electrophoresis of horse spleen apoferritin.
A horizontal SDS-PAGE (15% T, 5% C) was set up by using an LKB Multiphor electrophoresis system, the origin being made by using a Bio-Rad coaxial gel puncher of 2.5 mm diameter. The sample, prepared for electrophoresis in the absence of thiol was electrophoresed in the first dimension, and then, after treatment of the gel with 10% (v/v) 2-mercaptoethanol, electrophoresed at right angles to the first dimension.

treatment with thiol and ii) the 15,000 molecular weight species represents subunit with an intact intramolecular disulphide bond as witnessed by its conversion from an apparent molecular weight of 15,000 into the 19,000-molecular weight species on treatment with 2-mercapto-ethanol.

These disulphide bonds could have arisen as artifacts caused by, for example, oxidation of cysteine residues by dissolved oxygen in the presence of trace metal ions which were able to form disulphide linkages during the unfolding process, or they may have been present in the native apoferritin subunit. Clegg *et al* (1980) have suggested two alternative conformations of horse spleen apoferritin. On the basis of the sequence data of Heusterpreute and Crichton (1981) the first of these two conformations places the two cysteine residues at locations where formation of an intramolecular disulphide bond would be unlikely whereas the alternative subunit conformation A-C-D-P-E-B-L shows that such disulphide bonds are structurally possible.

Further evidence for the existence of an intramolecular disulphide bond within the subunit has come from gel permeation chromatography. Horse spleen apoferritin was dissociated into its subunits by treatment with 6M guanidine hydrochloride at 37 C for 2 hrs. This material was then applied to a column of Sephacryl S-200 superfine

equilibrated with 6M guanidine hydrochloride. The elution profile of this sample can be seen in Figure 30 The experiment was repeated under identical conditions using a sample of horse spleen apoferritin which had been treated with 40mM dithiothreitol in 6M guanidine hydrochloride. This elution profile is also shown in Figure 30 When these two profiles are superimposed one can see the effect of dithiothreitol treatment. The shape of the subunit elution peak is altered by dithiothreitol and under these conditions more material is eluted at an earlier retention time. This would indicate that dithiothreitol has reduced a disulphide bond within the subunit molecule giving it a less compact shape which would result in the material eluting earlier. This data provides further evidence for the existence of an intact disulphide bond within the subunit of horse spleen apoferritin.

The effect of thiol can also be seen when human ferritins from a variety of sources are electrophoresed. Figure 31 shows samples of human heart, liver and placental ferritins which were electrophoresed using a gradient pore polyacrylamide SDS gel with the concentration of 2-mercaptoethanol ranging from 0-10% (v/v). It can be clearly seen from these photographs that two subunit bands are present. However on addition of 2-mercaptoethanol there is a transition from the lower molecular

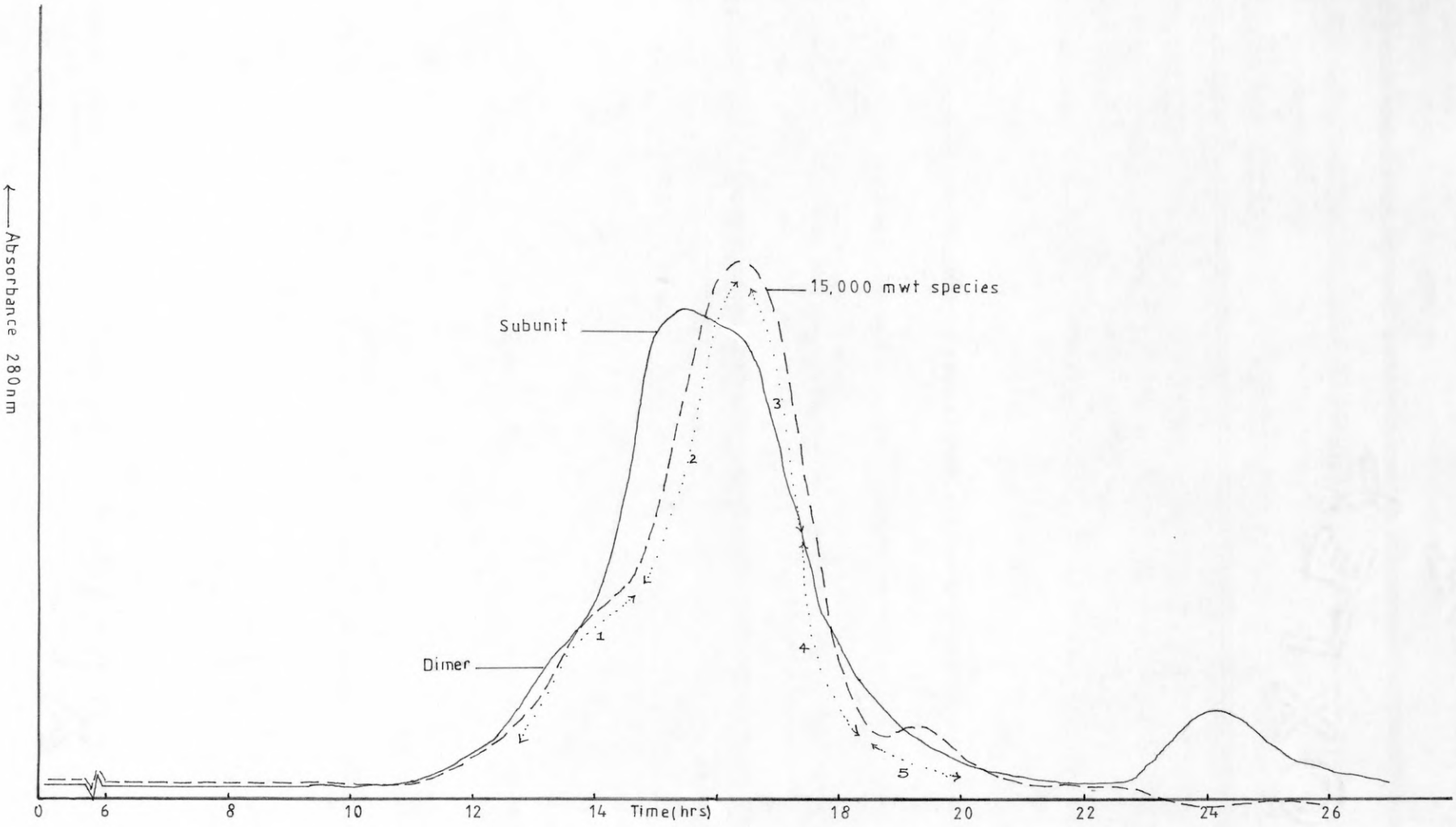


Figure 30: Gel permeation chromatography of horse spleen apoferritin in the presence (——) and absence (---) of dithiothretiol (40mM).

A sample of horse spleen apoferritin, prepared in the presence of 6M guanidine hydrochloride, was applied to a column of Sephacryl S-200 superfine (95 cm x 2.5 cm) flow rate.

←···x···→ indicates fractions which were pooled and futher investigated by SDS-PAGE (see page 124).

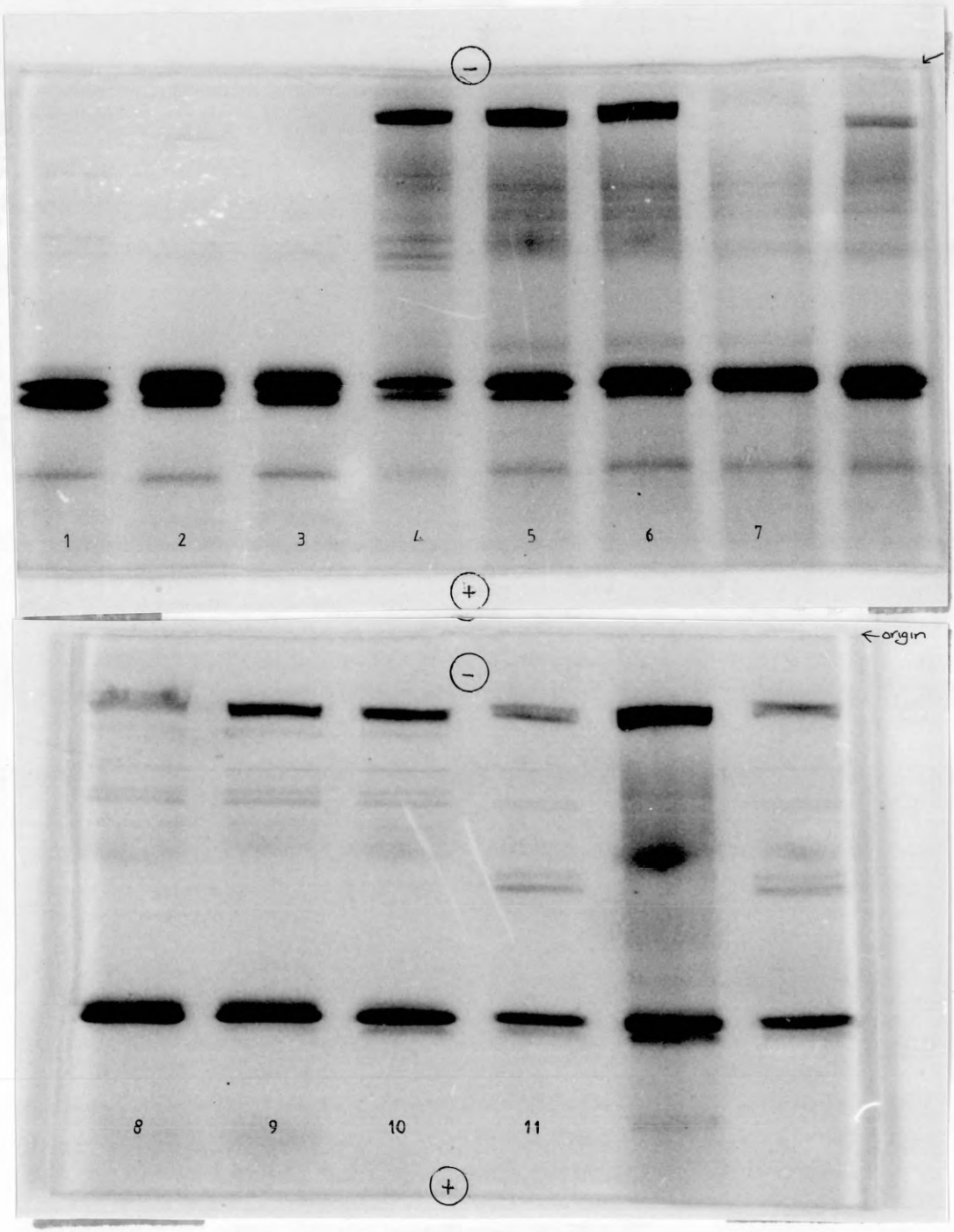


Figure 31: Electrophoresis of human heart, liver and placental ferritins in gradient-pore SDS polyacrylamide gel, 6-25%T, 5%C, pH 8.8.

Lane	1	Human heart ferritin	0%	(v/v)	2 mercaptoethanol
	2	" " "	1%	"	"
	3	" " "	10%	"	"
	4	Human kidney ferritin	0	"	"
	5	" " "	1	"	"
	6	" " "	5	"	"
	7	" " "	10	"	"
	8	Human placental ferritin	10	(v/v)	2 mercaptoethanol
	9	" " "	5	"	"
	10	" " "	1	"	"
	11	" " "	0	"	"

weight subunit band to the higher molecular weight subunit band.

The reasons for this transition are uncertain. It may be that, like horse spleen apoferritin, there is an intact disulphide bond which reduces the apparent molecular size of the subunit. Alternatively it may be an artifact caused by high concentrations of 2-mercapto-ethanol. It was noticed during the two-dimensional electrophoresis experiment that the second dimension (where 10% 2-mercaptoethanol was present) ran more slowly than the first dimension. Even if this transition is an artifact the two subunit bands appear to be real.

ii) Peptides arising from a specific proteolytic cleavage

When horse spleen apoferritin is electrophoresed in the presence of mercaptoethanol two stained bands consistently appear which have molecular weights below that of the subunit. Ishitani, Niitsu and Listowsky (1975) have estimated the molecular weights of these peptides, termed B and C to be 11,000 and 8,000 respectively.

Evidence suggests that these peptides arise from specific proteolytic cleavage, as incorporation of a protease inhibitor such as phenyl methyl sulphonyl fluoride to the isolation procedure significantly reduces their

appearance. These peptides have probably arisen as the result of a specific cleavage of the molecule either in an exposed region of the molecule or where two adjacent amino acids are susceptible to hydrolysis by virtue of their sequence. Heusterpreute and Crichton (1981) have found that the peptide bond between asp-pro at position 122-123 is easily cleaved by formic acid at 37°C.

It was decided to isolate these peptides in order to determine their origin within the subunit. As the cleavage site is most likely to occur on an exposed region of the subunit molecule, the identification of this site may assist in assignment of the amino acid sequence to the electron density maps.

To isolate peptides B and C a sample of horse spleen apoferritin which had previously been incubated in 6M guanidine hydrochloride was passed down a Sephacryl S-200 superfine column equilibrated with 6M guanidine hydrochloride. Alternatively material was isolated from a preparative gradient pore polyacrylamide gel containing SDS using an LKB Uniphor system. Fractions from the gel and column were characterized by electrophoresis in gradient-pore SDS polyacrylamide gels.

The molecular weight of peptide C has previously been reported to be 8,000 (Ishitani, Niitsu and Listowsky, (1975)) however on re-investigation using gradient-pore

polyacrylamide gels the molecular weight appears to be lower than previously thought. When a sample of horse spleen apoferritin is electrophoresed with molecular weight markers including the following:

Insulin Chain B	3,400
Aprotinin	6,500
Cytochrome c	12,500
Myoglobin	17,200
Lactate dehydrogenase	35,000

it can be clearly seen that the stained band representing peptide C lies between those of insulin chain B and aprotinin (see Figure 32). Likewise that of peptide B lies just below that of cytochrome c. When the methods of Lambin, Rochu and Fine (1976) and Poduslo and Rodbard (1980) were applied to this data the molecular weight of peptide C was estimated to be 4,500 while that of peptide B was in agreement with that of Ishitani, Niitsu and Listowsky at 11,000.

A sample of pure peptide C, as judged by SDS electrophoresis (see Figure 28 , lane 5), was taken for amino acid analysis and sequencing studies. The amino acid composition of this fraction was determined to be:

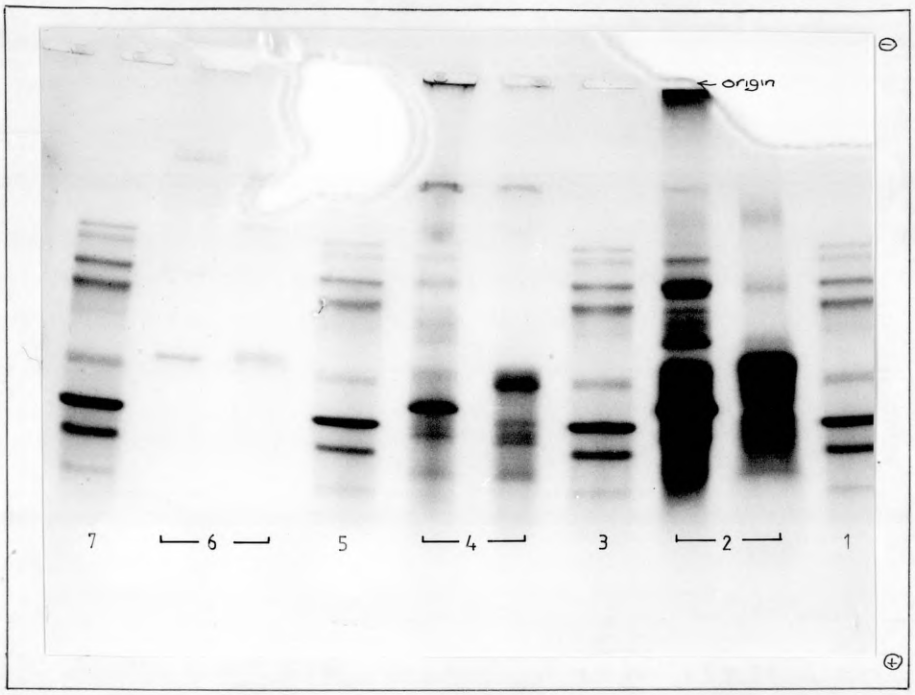


Figure 32: Molecular weight determination of peptide B and peptide C by gradient-pore SDS polyacrylamide gel electrophoresis, 6-25%T, 5%C, pH 8·8.

Lanes 1,3,5,7	Molecular weight markers	
	i) Aprotinin	3,400
	ii) Insulin Chain B	6,500
	iii) Cytochrome C	12,500
	iv) Myoglobin	17,200
	v) Lactate dehydrogenase	35,000
Lanes 2,4,6	Horse spleen apoferritin, in each case the left hand track is electrophoresed in the absence of thiol.	

Asx	5.5	Ala	4.0	Tyr	0.9
Thr	2.2	Val	1.8	Phe	1.5
Ser	2.6	Met	0.9	His	1.7
Glx	5.4	Ile	0.9	Lys	2.5
Pro	0.9	Leu	5.0	Arg	2.4
Gly	4.0				

This composition suggests that peptide C has arisen from a proteolytic cleavage in the C-terminal region of the protein since the N-terminal region is known to be rich in phenylalanine and tyrosine. Using liquid-phase Edman degradation in a Beckman 890 Sequencer the first six amino acids in the sequence of peptide C were determined to be:

Leu-thr-leu-lys-aromatic-trp-

This sequence work was carried out by Monsieur P. Talmagne at the Université d'Etat à Mons, Belgium, during a two month FEBS fellowship at the Université Catholique, Louvain-la-Neuve, Belgium.

Comparison of this with the published sequence of horse spleen apoferritin shows that it corresponds to the C-terminal region of the protein and would suggest that peptide C has arisen as a result of a specific cleavage of a heavier subunit. If one looks at the two alternative conformations of the apoferritin subunit proposed by P. Harrison and colleagues (see Figure 8) the cleavage site for this peptide would be either at the

end of helix E or at the end loop L, both of which are exposed regions of the subunit where proteolysis may occur. It is possible that the subunit L has arisen from proteolytic cleavage of the heavier subunit H i.e. L + C = H. If the molecular weights of subunit L and peptide C are added (subtracting the overlapping region) the overall molecular weight is 23,500. This is higher than the molecular weight of the subunit proposed by Drysdale. This suggestion seems unlikely on two accounts. Firstly post-translational modification of proteins is often an N-terminal event and if the subunit H were a precursor of L one would expect the sequence of peptide C to have no correlation with that of the L subunit. Secondly recent evidence from Otsuka, Maruyama and Listowsky (1981) based on the amino acid composition of H + L subunits from horse liver apoferritin suggest that they are separate gene products as subunit H contains fewer leucine, phenylalanine alanine and arginine residues than subunit L.

Further work on peptide C is therefore necessary to determine its location within the primary structure and its role, if any, in the conformational structure and function of horse spleen apoferritin.

iii) The glycosylation of subunits

Recent evidence suggests that the microheterogeneity of ferritin which is apparent on isoelectric focusing may,

in part, be due to glycosylation of the subunit rather than varying proportions of H and L subunits. It has been shown that treating human serum ferritin with neuraminidase substantially reduces the presence of acidic isoferritins on isoelectric focusing presumably due to the release of sialic acid residues (Cragg, Wagstaff and Worwood, 1980). The presence of a glycosylated subunit in human serum ferritin has also been detected in polyacrylamide gels containing SDS with a molecular weight of 23,000, 'G' subunit (Cragg, Wagstaff and Worwood, 1981).

The presence of a glycosylated subunit of horse spleen ferritin was investigated using an affinity column for glycoproteins. In this way it was hoped to isolate a sample of glycosylated ferritin from which further studies could be carried out. Glycogel-B contains boronate ligands immobilized on cross-linked beaded agarose. This support medium was used to retain glycosylated ferritin and separate it from the non-glycosylated ferritin. As a result of the work carried out investigating the effect of thiol concentration it was thought that a small proportion of material attached to the column was non-glycosylated but remained attached to the support via disulphide linkages to a glycosylated subunit. This was in fact shown to be the case as a fraction was eluted by incorporating 1% (v/v) 2-mercaptoethanol into the starting

buffer (0.25M ammonium acetate pH 8.5). Due to the absorbance at 280nm of 2-mercaptoethanol the column was re-equilibrated in starting buffer until the base-line had been re-established then glycosylated ferritin was finally eluted using a 0.2M sorbitol pH 8.5.

Figures 33 and 34 show the elution profile obtained using the Glycogel B column and the corresponding SDS polyacrylamide gel. The three fractions, non-glycosylated, non-glycosylated s-s linked and glycosylated ferritin showed no appreciable structural differences when electrophoresed in polyacrylamide SDS gels. This would imply that although glycosylation had occurred it was insufficient to alter the molecular weight of the subunit, however the post-transitional addition of sugars may account for the occurrence of some isoferritins on isoelectric focusing.

Glycosylation may not only modify the properties of ferritin but may also regulate the catabolism of the glycoprotein. Worwood *et al* (1979) have suggested that a small proportion of nascent ferritin enters the plasma after synthesis on polysomes bound to endoplasmic recticulum, the ferritin being glycosylated in the process. The glycosylation of ferritin may influence the rate of removal of ferritin from the plasma so that glycosylated ferritin is cleared more slowly than non-glycosylated ferritin. Zaman and Veilwikghen (1981) however found that

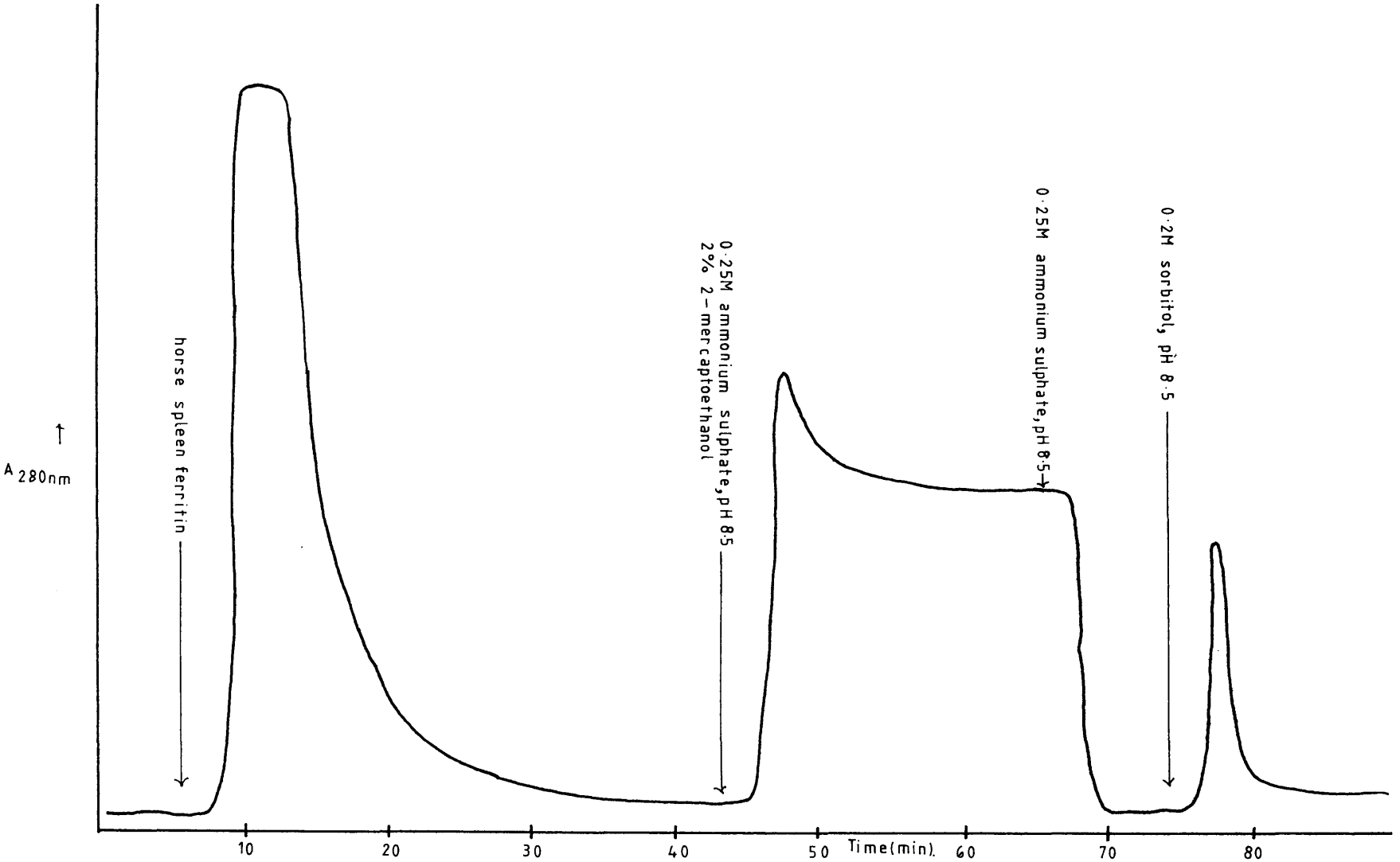


Figure 33: Elution of horse spleen apoferritin using an affinity column for glycoproteins.

Glycogel B column equilibrated with 0.25M ammonium acetate pH 8.5
Non glycosylated ferritin was eluted with 0.25M ammonium acetate, pH 8.5
Non-glycosylated-S-S-linked ferritin was eluted with 0.25M ammonium acetate, pH 8.5, 1% (v/v) 2-mercaptoethanol
Glycosylated ferritin was eluted with 0.2M sorbitol, pH 8.5
Flow rate 0.5 ml/min

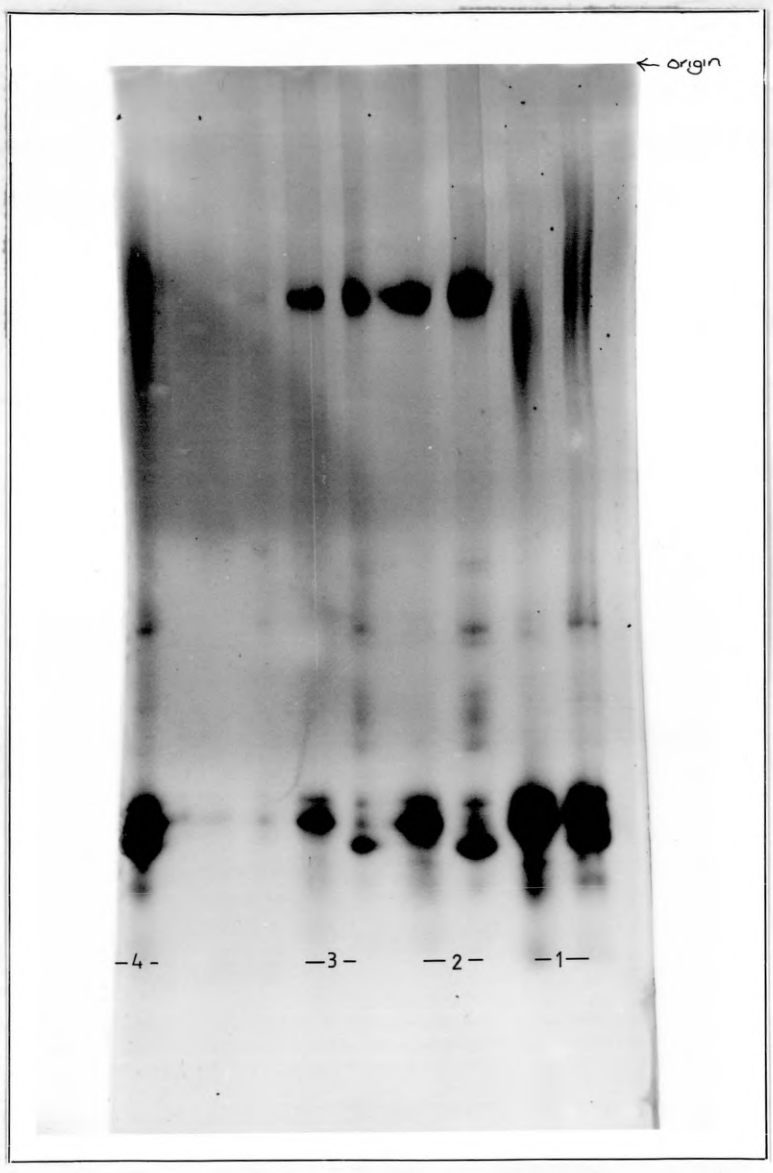


Figure 34: Electrophoresis of glycosylated and non-glycosylated horse spleen ferritin.

Lane 1 Non-glycosylated ferritin
2 Non-glycosylated-s-linked ferritin
3 Glycosylated ferritin
4 Untreated ferritin

The samples were applied to a 6-25%T, 5%C, SDS polyacrylamide gel, pH 8·8.
The samples were prepared in 0·125M Tris buffer, pH 6·8 containing 1% (w/v) SDS. In each case the samples in the left hand tracks additionally contained 1% (v/v) 2-mercaptoethanol.

glycosylated (or mannosylated) and non-glycosylated rat liver ferritin, when injected into rats via the tail vein, had identical plasma half-lives.

These workers also found that glycosylation and mannosylation of horse spleen ferritin and rat liver ferritin could occur non-enzymatically. They have shown that the incorporation of carbohydrates *in vitro* into ferritin depended upon the concentration of both carbohydrates and ferritin.

This implies that newly synthesized ferritin would be devoid of carbohydrate compared to the older molecules. Whether non-enzymic glycosylation would occur *in vivo* has yet to be established however there is strong evidence to support the fact that varying degrees of glycosylation accounts for the occurrence of some isoferritins.

Cleavage at Tryptophan Residues Using o-Iodosobenzoic acid

The tryptophan content of horse spleen apoferritin has been estimated by the spectrophotometric methods of Edelhoch (1967) and Bencze and Schmid (1957) and by chemical modification using 2-nitrophenylsulphenyl chloride (Boccu *et al*, 1970) to be 2 residues per subunit (Bryce and Crichton, 1971). This result is in disagreement with the sequence data of Heusterpreute (Heusterpreute and Crichton, 1981) who found only one tryptophan at position 89. It was decided to re-assess the number of tryptophan residues by cleaving horse spleen apoferritin subunits at the carboxyl side of tryptophan using o-iodosobenzoic acid. Another reason for carrying out this cleavage method was to complement the second derivative spectrophotometric studies.

Results

O-Iodosobenzoic acid was used in this experiment as it is a mild reagent which selectively cleaves proteins and peptides at the carboxyl side of tryptophan residues leaving a free amino group and a C-terminal spirolactone.

The material used in this experiment was a preparation of horse spleen apoferritin which had been further purified by preparative electrophoresis. When examined on SDS gradient-pore polyacrylamide gels, in the presence of 2-mercaptoethanol, this gave a single subunit band with

a molecular weight of 18,500-19,000 (see Figure 35, lanes 3-5) After reduction and carboxymethylation of cysteine residues the protein was cleaved by the method of Mahoney and Hermodson (1979) using o-iodosobenzoic acid.

This chemically digested material was analyzed on a gradient pore SDS polyacrylamide gel. The results, as shown in Figure 35, show that the cleavage reaction was complete as no subunit material appeared to be present. Only one band was visible with a molecular weight of approximately 9-10 thousand. This result agrees well with the sequence data of Heusterpreute and Crichton (1981) as cleavage after tryptophan 89 would yield two peptides, an N-terminal peptide with 89 residues and a C terminal peptide with 85 residues. These two peptides would be indistinguisable on SDS gradient pore electrophoresis.

As the N-terminal end of the subunit protein is acetylated it is therefore blocked to sequential cleavage by Edman degradation. It was decided to sequence this material directly as the only free α-amino group available for reaction with phenylisothiocyanate would be that from amino acid 90. The cleaved material was attached to an aminopropyl glass support using p-phenylenediisothiocyanate, a reagent which covalently attaches the peptide to the support material via the α-amino group and the ε amino groups of lysine. A total of 13 sequence cycles were carried out. After conversion

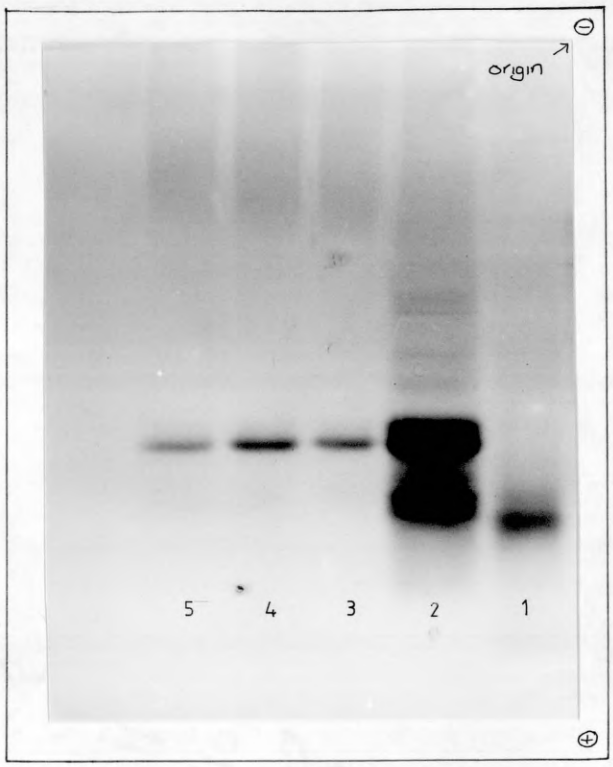


Figure 35: Chemical digestion at tryptophan residues using o-iodosobenzoic acid.

Gradient pore SDS polyacrylamide gel (6-25% T, 5%C, pH 8.8)
Lane 1 Chemically digested material
2 Unpurified ferritin
3-5 Purified ferritin
Material in lanes 3-5 was used for cleavage with o-iodosobenzoic acid.

of the anilinothiazolinone amino acids to their phenylthiohydantoin (PTH) derivatives they were analyzed by thin layer chromatography. The results are shown in Figure 36 and summarized below.

Sequence of Heusterpreute	^{89}trp	glu	thr	thr	leu	asp	ala	met	lys	ala	ala	^{100}ile
Sequence cycle		1	2	3	4	5	6	7	8	9	10	11
Identification								ala		ala		

It seems unusual that only two PTH amino acids are identifiable, alanine at cycle 7 and alanine at cycle 9. These results were confirmed by hplc against standard PTH amino acids. If the sequence of Heusterpreute and Crichton is correct then cycles 1 and 8 would be retained on the support, also the anilinothiazolinones of threonine (cycles 2 and 3) are very unstable making these amino acids difficult to detect. If one compares this section of the protein with the published sequences of human spleen apoferritin (Wustefeld and Crichton, 1982) it is apparent that this region shows considerable variation in its amino acid composition

		^{90}									^{100}		
i)	~Trp	gly	thr	thr	leu	asp	ala	met	lys	ala	ala	ile	val~
ii)	~			lys		pro						met	ala~
iii)	~			ser	gly	leu	asx			glu	cys	leu	his~

i) sequence of horse spleen ferritin

ii) sequence of human spleen ferritin (major component)

iii) sequence of human spleen ferritin (minor component).

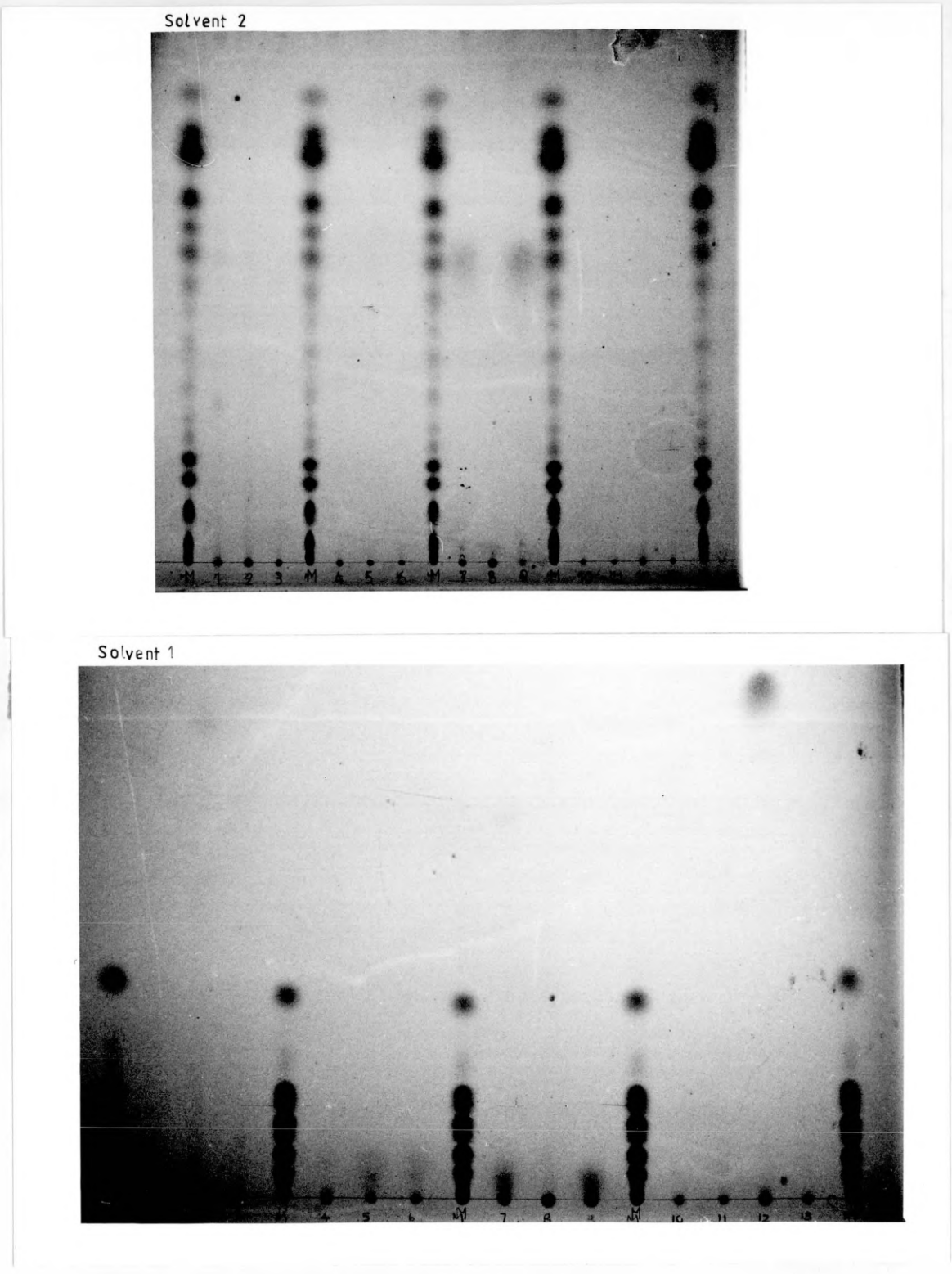


Figure 36: Thin layer chromatography of PTH amino acids obtained from sequencing horse spleen apoferritin which had been cleaved at tryptophan residues.

Cycles 7 and 9 indicate the presence of alanine.

It may be that this sequence has arisen from cleavage of a minor component and that the major peptide component is, for some reason, blocked to sequencing.

When a sample of peptide C (see earlier section) was sequenced the first six amino acids were found to be -leu-thr-leu-lys-aromatic-trp-. This sequence of amino acids corresponds well to the last six amino acids at the C terminal end of the published sequence of horse spleen apoferritin, -^{169}leu-thr-leu-lys-his-asp^{174}. From the molecular weight of peptide C ($\sim$4500) one would expect this sequence to extend beyond 174 amino acids as it is possible that peptide C has arisen as a result of a specific cleavage of a heavier subunit. The sequencing result from the cleavage with o-iodosobenzoic acid may represent a region from peptide C. Amino acid analysis of this peptide indicates that four alanine residues are present. As no small molecular weight peptides were detected on SDS gels this seems unlikely however there may only have been a small amount of material present which was undetectable by conventional staining procedures such as Coomassie Blue. If the gel had been stained by a more sensitive procedure such as silver staining this may have revealed small molecular weight peptide bands and also the presence of a heavier molecular weight subunit band from which the peptide may have originated.

Second Derivative Spectroscopy for Evaluating the Tyrosine to Tryptophan Ratio

When a sample of protein is hydrolysed for amino acid analysis little or no tryptophan is normally detected. The principle reason for this is that the standard procedure of hydrolysis in 6M HCl results in the destruction of tryptophan. Although methods have been developed to increase the yield of tryptophan, for example the addition of mercaptans to 6N HCl (Matsubara and Sasaki, 1969) and replacing HCl with p-toluenesulphonic acid (Liu and Chang, 1971), none of these methods give consistently satisfactory results. Hydrolysis may also be carried out using alkaline conditions which are less destructive towards tryptophan. Hugh and Moore (1972) have developed a method using NaOH in the presence of starch which increases the recovery of tryptophan, but even under these milder conditions the yields are not normally quantitative.

The problems associated with hydrolysis may be overcome by determining the tryptophan content of intact proteins rather than their hydrolysates. Analysis may be carried out spectrophotometrically to determine not only the tryptophan content but also the tyrosine content of proteins. Direct spectroscopic measurements in the near u.v. region (250nm - 300nm) can provide

information on the tryptophan and tyrosine content of proteins. However in this region of the spectrum there is a strong degree of overlap between the absorption bands of these two chromophores which must be accounted for in any calculations. Some of the techniques used to determine tyrosine and tryptophan take advantage of the different absorption maxima of tyrosine and tryptophan in strongly alkaline solution (Bencze and Schmid, 1957) or are based on the absorption measurements at two or more wavelengths in denaturing solvents at neutral pH (Edelhoch, 1967). All these methods need high protein concentrations and are considerably affected by the presence of u.v. absorbing components including phenylalanine, cysteine and prosthetic groups.

The use of second derivative spectroscopy enables the contributions of tyrosine and tryptophan to be isolated from the complex absorption spectrum of a protein and for their relative ratios to be determined. The spectral bands of these two chromophores are only partly resolved using second derivative spectroscopy but the interference of tyrosine on the tryptophan spectrum can be shown to be related (although not linearly) to the tyrosine to tryptophan ratio in both model compound mixtures and proteins. This technique was used to determine the tyrosine and tryptophan content of a subunit of horse spleen apoferritin.

N-acetyl-L-tryptophanamide and N-acetyl-L-tyrosinamide were chosen as model compounds to represent tyrosyl and tryptophanyl residues in proteins. Typical second derivative spectra of these model compounds are shown in Figure 37. From these traces one can clearly see how the spectral bands of these two major protein chromophores overlap to a large extent in this region of the spectrum. Servillo *et al* (1982) have been able to demonstrate that this mutual interference between the second derivative bands of tyrosine and tryptophan is related to the molar concentrations of these two amino acid residues.

The spectrum of N-acetyl-tryptophanamide is characterised by two minima at 282.5nm and 290.5nm and two maxima at 287nm and 295nm. In the same spectral region N-acetyl-tyrosinamide shows a single minimum at 283.5nm and two maxima at 286nm and 289.5nm. When the second derivative spectrum of a protein, or a mixture of the two model compounds, is taken it resembles the spectrum of N-acetyl-tryptophanamide more closely. This is a result of the higher intensity of the tryptophanyl bands as compared to the tyrosinyl bands. However the amplitude of the spectral bands are strongly affected by tyrosine. The peak-to-peak distance between the maximum at 295nm and the minimum at 290.5nm (indicated as distance (b) in Figure 37) is decreased at high tyrosine/tryptophan ratios because the tyrosyl maximum at 289.5nm reduces

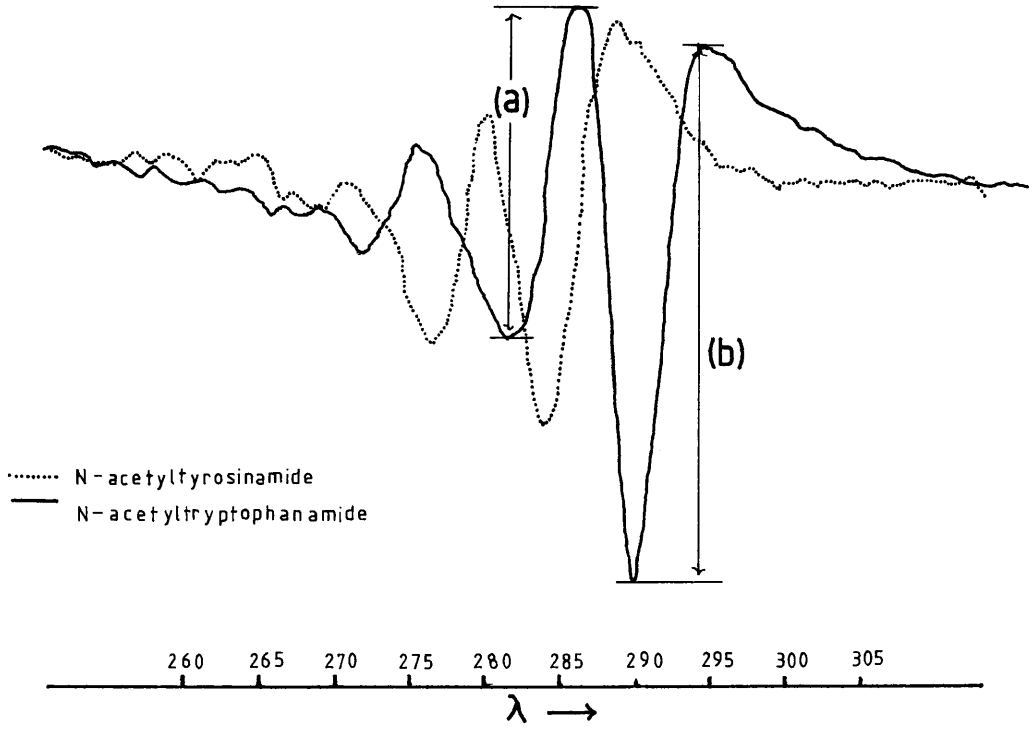


Figure 37: Second derivative spectra of N-acetyl-tryptophanamide and N-acetyl-tyrosinamide.

(a) represents the peak-to-peak distance between the maximum at 287 nm and the minimum at 283 nm.

(b) represents the peak-to-peak distance between the maximum at 295 nm and the minimum at 290.5 nm.

the intensity of the tryptophanyl minimum at 290.5nm. The peak-to-peak distance between the maximum at 287nm and the minimum at 283nm (indicated as distance (a) in Figure 37) is increased at high tyrosine/tryptophan ratios as both chromophores give a minimum in this region, thus enhancing the overall negative peak. The model compounds N-acetyl-tyrosinamide and N-acetyl-tryptophanamide were used to determine the dependence of the ratio r = (a)/(b) on the molar ratio of tyrosine to tryptophan. The results of these experiments are listed in Table 7 and shown graphically in Figure 38 From these results the value of r can be seen to be a function of x, the molar ratio of tyrosine/tryptophan.

Typical second derivative spectra are shown in Figure 39 for dissociated horse spleen apoferritin and completely unfolded subunits of apoferritin. Close examination of these results reveal how the spectrum changes as the tyrosyl and tryptophanyl residues in the protein become exposed to solvent during the unfolding procedure. Most of the tyrosine residues in horse spleen apoferritin are thought to be located at subunit-subunit interfaces or buried within the protein shell, whereas it is suggested that tryptophan residue(s) are on the surface of the protein but may only be partially exposed. The effect of the perturbation of tyrosine residues on the spectrum of tryptophan can clearly be seen by a reduction in the value of (b), the peak-to-peak distance

Total Volume (ml)	Additions of N-Ac-Trp* (µl)	Additions of N-Ac-Tyr** (µl)	(a) (mm)	(b) (mm)	r=(a)/(b)	[N-Ac-Trp] x 10^{-5}	[N-Ac-Tyr] x 10^{-5}	$\frac{[\text{N-Ac-Tyr}]}{[\text{N-Ac-Trp}]}$
2.600	100	-	56.75	92.00	0.617	12.20	0	0
2.625	-	25	62.00	87.50	0.709	12.08	2.86	0.238
2.650	-	25	65.00	83.00	0.783	11.97	5.67	0.474
2.675	-	25	60.00	80.25	0.872	11.86	8.43	0.711
2.700	-	25	74.50	77.75	0.953	11.75	11.14	0.960
2.725	-	25	79.00	75.00	1.053	11.64	13.79	1.185
2.750	-	25	85.00	72.50	1.1724	11.53	16.40	1.421
2.775	-	25	91.25	69.25	1.3177	11.43	18.96	1.659
2.800	-	25	94.00	67.00	1.4030	11.33	21.47	1.896
2.825	-	25	97.25	66.25	1.4680	11.23	23.95	2.133
2.850	-	25	100.75	64.50	1.562	11.08	26.37	2.380
2.875	-	25	108.50	60.25	1.800	11.03	28.76	2.610
2.900	-	25	112.75	60.00	1.929	10.94	31.10	2.844
2.950	-	50	122.25	54.00	2.264	10.75	35.67	3.318
3.000	-	50	133.00	51.25	2.595	10.57	40.09	3.792
3.050	-	50	145.25	46.50	3.124	10.40	44.36	4.266
3.100	-	50	158.25	43.25	3.665	10.23	48.49	4.740
3.150	-	50	168.50	38.25	4.405	10.07	52.49	5.214
3.200	-	50	179.25	35.00	5.121	9.91	56.38	5.687
3.250	-	50	183.75	32.50	5.654	9.76	60.13	6.162
3.300	-	50	197.25	29.25	6.755	9.61	63.78	6.636
3.350	-	50	203.25	26.00	7.818	9.47	67.31	7.170
3.400	-	50	213.00	23.25	9.161	9.33	70.75	7.584
3.450	-	50	216.25	20.75	10.422	9.19	74.08	8.057
3.500	-	50	223.25	16.75	13.328	9.06	77.31	8.531

* N-Ac-Trp ≡ N-acetyl-tryptophanamide
** N-Ac-Tyr ≡ N-acetyl-tyrosinamide

Table 7: Dependence of the ratio r on the molar ratio of N-acetyl-tyrosinamide/N-acetyl-tryptophanamide

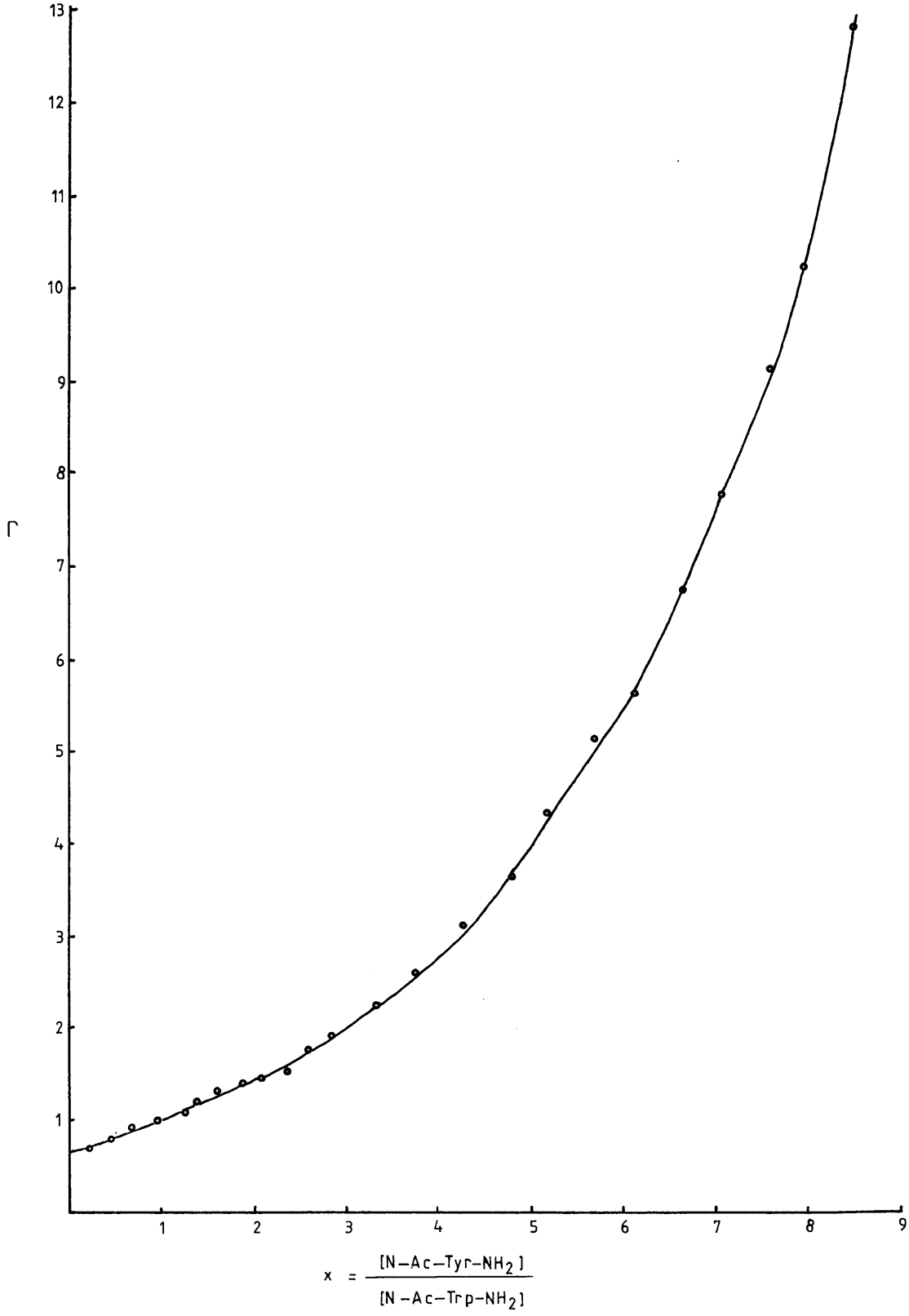


Figure 38: The dependence of the ratio r on the molar ratio of N-acetyl-tyrosinamide to N-acetyl-tryptophanamide.

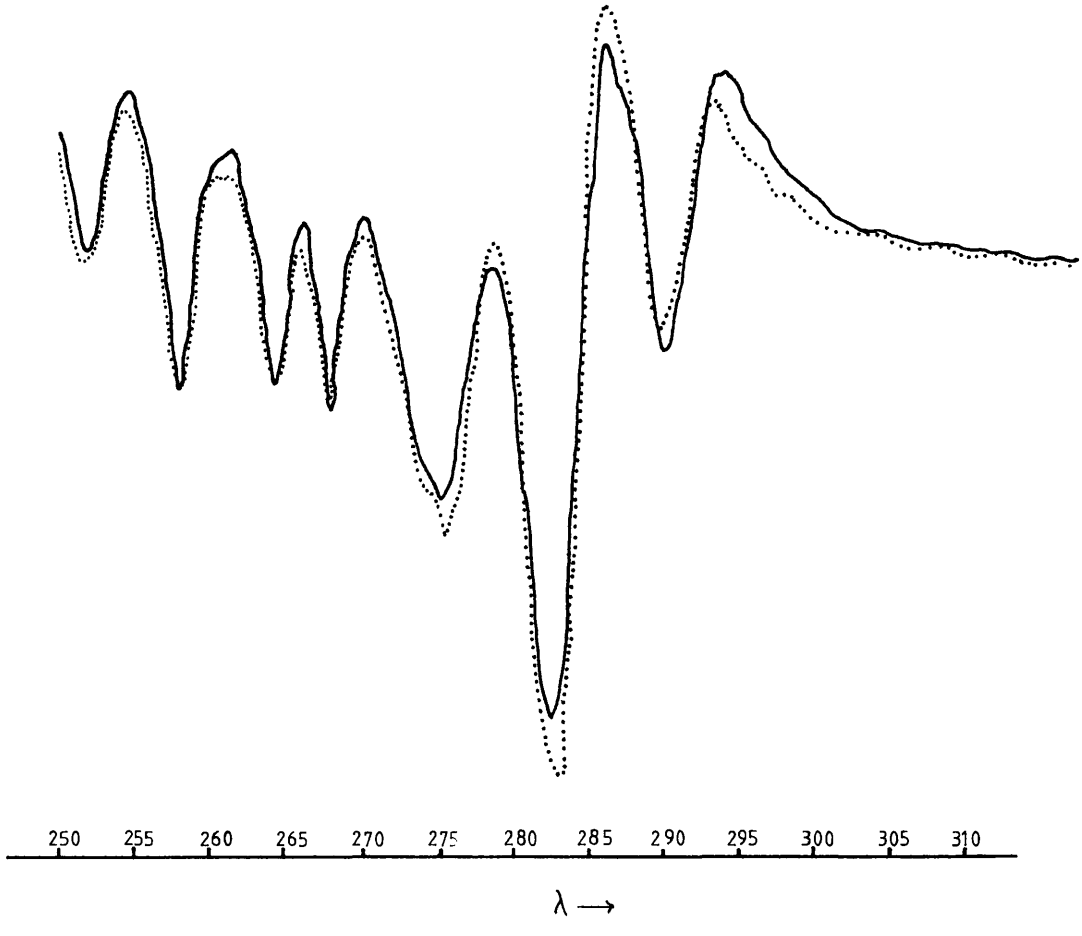


Figure 39: Typical second derivative spectra of horse spleen apoferritin.

i) Spectrum taken after 10 seconds (——)
ii) Spectrum taken after 140 min (·····)

ii) Represents fully dissociated subunits

at 290.5/295nm and an increase in the value of (a), the peak-to-peak distance at 283/287nm. This is due to the spectral bands of tyrosine and tryptophan opposing each other in the region of the spectrum where (b) is measured thus causing a reduction in distance (b), whereas they complement each other in the region where (a) is measured resulting in an increased value of distance (a).

Using the relationship shown in Figure 38 it was possible to estimate the molar ratio of tyrosine to tryptophan in samples of dissociated horse spleen apoferritin subunits. This was found to be 5.2 ± 0.15 (an average of 10 separate determinations) indicating that there are 5-6 tyrosine residues per subunit and one tryptophan residue per subunit, which is in good agreement with the sequence data of Heusterpreute and Crichton (1981). This study only provided information on the molar ratios of these amino acids rather than the absolute values. To achieve the latter required the use of second derivative spectrophotometric titration methods.

Determination of the Absolute Molar Content of Tryptophan

Second derivative spectrophotometric titration were performed by adding aliquots of the model compound N-acetyl-tyrosinamide to a solution of dissociated horse spleen apoferritin subunits. The molar ratio, r, was

determined after each addition and using the calibration curve in Figure 38 the value of x, the molar ratio of N-acetyl-tyrosinamide/N-acetyl-tryptophanamide, was obtained.

Servillo *et al* (1982) found that the value of x, upon each chromophore addition, is related to the initial protein concentration Cp by

$$x = \frac{n_{tyr}\, C_p + C_{tyr}}{n_{trp}\, C_p}$$

which reduces to

$$x = x_o + \frac{C_{tyr}}{n_{trp}\, C_p}$$

where x_o is the initial tyr/trp ratio before each addition

n_{tyr} the number of tyrosine residues contained in the protein

n_{trp} the number of tryptophan residues contained in the protein

C_{tyr} the amount of N-acetyl-tyrosinamide added to the solution

C_p the initial protein concentration.

When the value of x is plotted against C_{tyr} the relationship is found to be linear as shown in Figure 40 and the slope of this line is equal to $\frac{1}{n_{trp}\, C_p}$.

The absolute molar value of tryptophan can therefore be calculated from this relationship. For a sample of horse spleen apoferritin subunits this was found to

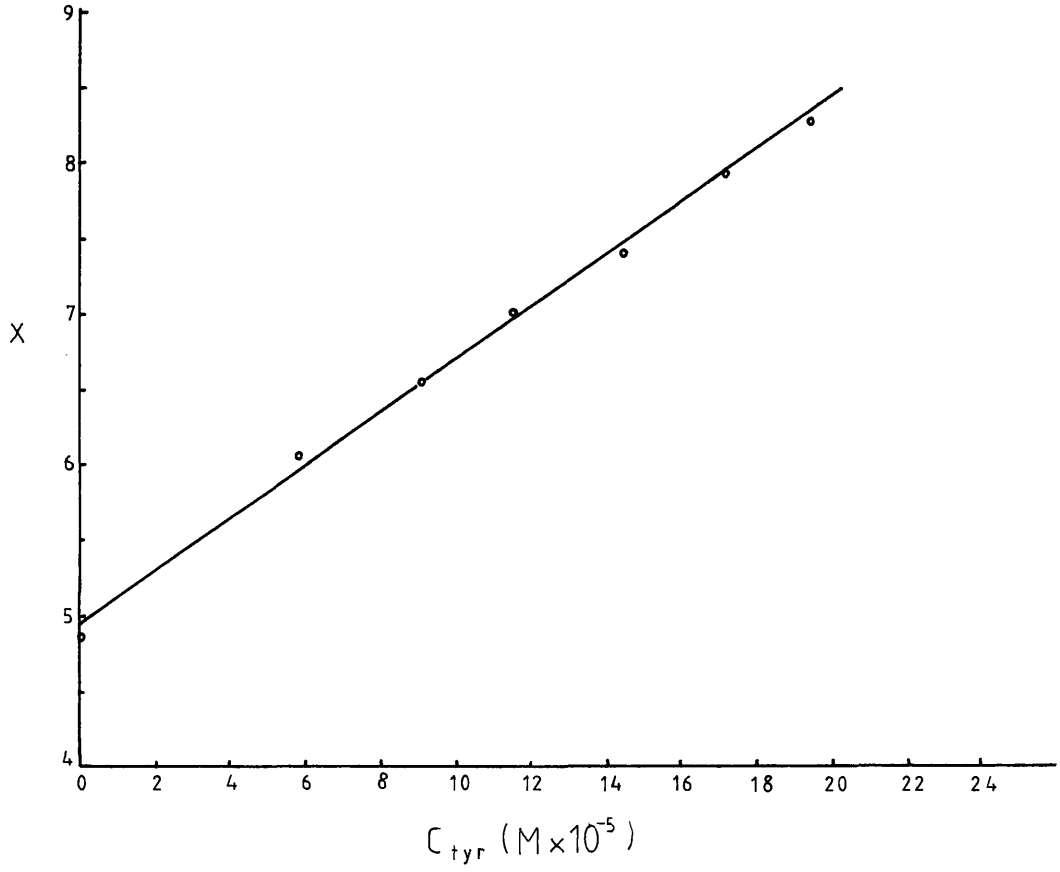


Figure 40: Determination of absolute molar content of tryptophan residues in horse spleen apoferritin by second derivative spectrophotometric titration.

contain 1.42 ± 0.15 tryptophan residues per subunit (average of 10 determinations) by standard deviation.

Determination of the Absolute Molar Content of Tyrosine

Servillo *et al* (1982) have recommended the use of this titration procedure for proteins with high tyrosine to tryptophan ratios.

Second derivative spectrophotometric titrations were carried out using the same procedure as described for the determination of the molar content of tryptophan only in this case aliquots of N-acetyl-tryptophanamide were added to the solution. In this titration a plot of 1/x vs tryptophan concentration provides a straight line as shown in Figure 41 , the slope of which is $(1/n_{tyr})\,C_p$. From this relationship it was possible to calculate the absolute value for the number of tyrosine residues per subunit of horse spleen apoferritin and this was found to be 5.57 ± 0.12 (average of 10 determinations). by standard deviation.

In all these estimations the molecular weight of a subunit of horse spleen apoferritin was taken as being 19,800, as calculated from the amino acid sequence of Heusterpreute and Crichton (1981).

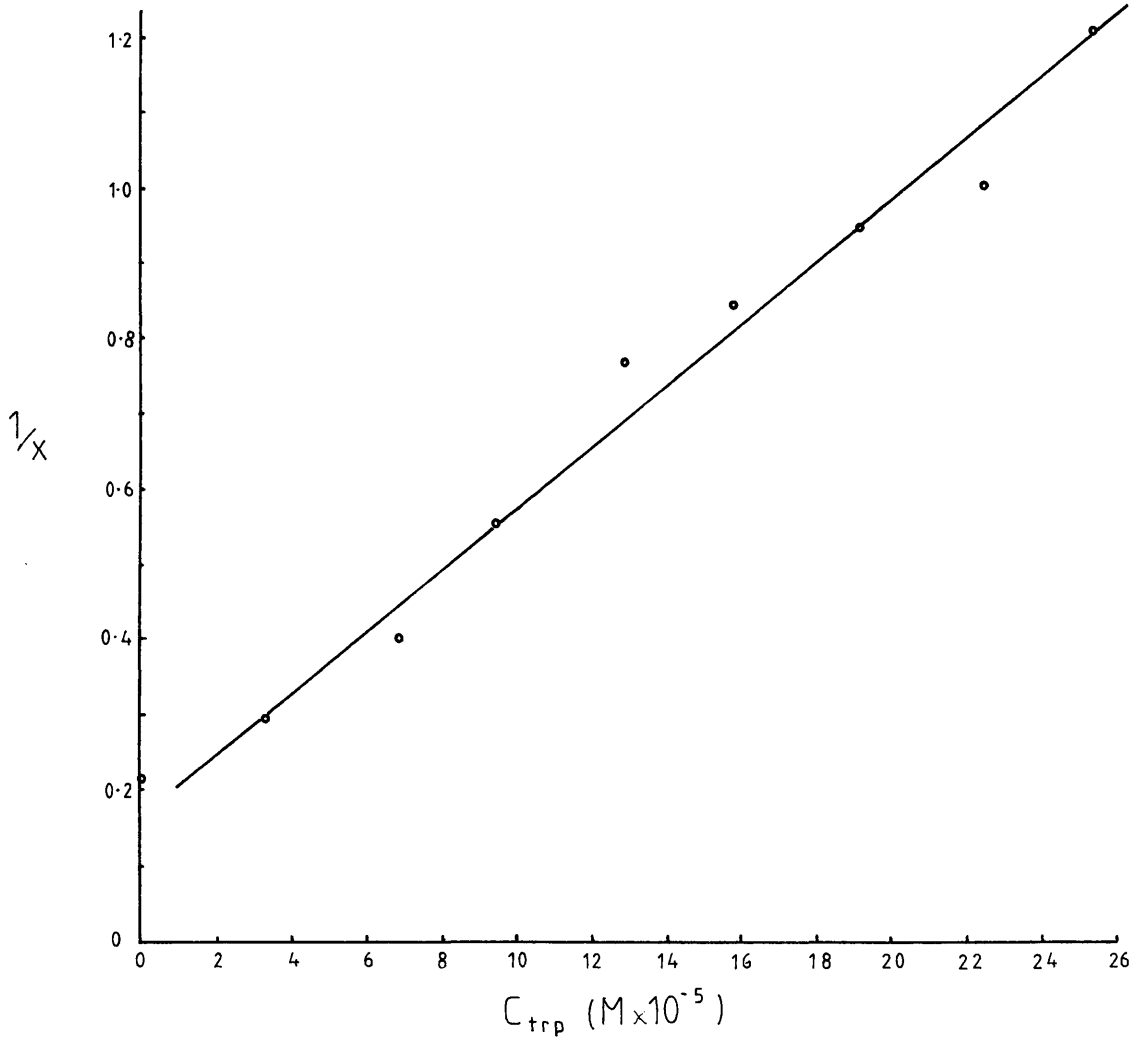


Figure 41: Determination of absolute molar content of tyrosine residues in horse spleen apoferritin by second derivative spectrophotometric titration.

Discussion

Early studies on the amino acid composition of horse spleen apoferritin indicated the presence of 5 tyrosine residues per subunit. These estimations were obtained initially from amino acid analysis (Bryce and Crichton, 1971) and later confirmed by chemical modification using tetranitromethane (Crichton and Bryce, 1973). At this time the tryptophan content was estimated by the spectrophotometric methods of Edelhoch (1967) and Bencze and Schmid (1957) and by chemical modification using 2-nitrophenyl sulphenyl chloride (Bryce and Crichton, 1971) and found to be 2 residues per subunit. The values of 5 tyrosine and 2 tryptophan residues per subunit appeared to be consistent with the known value of the specific extinction coefficient of $E^{1\%}_{1cm}$ (280nm) = 9.81 on the basis of the reported value for the molar extinction coefficients of tyrosine and tryptophan (Bryce and Crichton, 1973).

The possible location of these two amino acid residues within the apoferritin molecule were also investigated at this time. Ultra-violet difference spectroscopy was used to study the dissociation of the protein shell into its subunits (Crichton and Bryce, 1973). In this study they concluded that, on dissociation into subunits at low pH, 4 to 5 tyrosine residues/subunit were transferred from the interior of the protein into the solvent. In

addition to the involvement of tyrosyl residues during conformational changes at low pH, spectral evidence was presented for a change in the environment of tryptophanyl residues on dissociation. Using Hill plot treatment the perturbation of tryptophan was found to be a co-operative process involving at least two carboxyl groups. No co-operativity was found at alkaline pH. On alkaline dissociation all 5 tyrosine residues were titrated simultaneously with a pk_{app} of 11.9 (Crichton and Bryce, 1973). This high pk value for the tyrosine residues is probably due to their position in the protein shell. If they are located in the hydrophobic environment of subunit-subunit interfaces then one would expect an increased pk value relative to the pk of an exposed tyrosine residue.

Recently, on sequencing the horse spleen apoferritin subunit, Heusterpreute and Crichton (1981) demonstrated that the subunit only has one tryptophan at position 89 and 6 rather than 5 tyrosine residues, these being at position 8, 23, 28, 30, 36 and 164. As pointed out by these workers the fact that there was only a single tryptophan residue per subunit was difficult to reconcile with the known specific extinction coefficient and the earlier spectrophotometric studies, and it was for this reason that the present investigation was undertaken.

The results of the second derivative spectrophotometric titrations provide good evidence to support the view that there are indeed 6 tyrosine residues and one tryptophan residue per subunit, values which are in good agreement with the sequence data. That this is the case requires an explanation for the previously accepted value of the specific extinction coefficient.

As mentioned earlier the literature value for $E^{1\%}_{1cm}$ (280nm) = 9.81 appeared to be in good agreement with the known molar extinction coefficients for the 280nm absorbing amino acids, tyrosine, tryptophan, phenylalanine and cysteine if it was assumed that there were 5 tyrosine and 2 tryptophan residues per subunit. If the specific extinction coefficient is recalculated on the emergence of new values for tyrosine and tryptophan then $E^{1\%}_{1cm}$ (280nm) = 6.87, as compared to $E^{1\%}_{1cm}$ (280nm) = 9.81, the generally accepted specific extinction coefficient. Part of the difference between these two values may be attributed to the location of these residues within the quaternary structure of the protein. From earlier work it was demonstrated that at least 4 of the tyrosine residues are internal residues as is a single tryptophan residue (Crichton and Bryce, 1973). This view is further substantiated on the basis of the most recent X-ray crystallographic data of Bourne *et al* (1982a,b) who

suggest that the trp^{89}, although lying on the molecular surface of the protein, appears to be tucked into a surface pocket. Their data, and also the results of chemical modification (Silk and Breslow, 1976) suggest that 5 of the tyrosine residues (23, 28, 30, 36 and 164) are buried.

When we consider those factors which affect the absorption properties of a chromophore, we find that those uv-absorbing amino acids which are present in internal regions of a protein exhibit an increase in their molar extinction coefficient. In order to account for the difference in the specific extinction coefficient this increase would need to be approximately 30%.

Recently some workers have found a flavin-like co-factor associated with apoferritin (Mareschal *et al* (1980); Crichton *et al* (1980). In addition a fluorescent moiety is also thought to be attached in same way to horse spleen apoferritin. This component remains linked to the protein even after exposure to denaturants, dialysis and electrochromatography procedures (Maruyama and Listowsky, 1982). Both these components may contribute to the value of the specific extinction coefficient being higher than the calculated value. The presence of differing amounts of H subunit, with presumably a different amino acid composition, could also be a contributing factor.

Kinetics of Unfolding

An additional experiment was carried out to investigate the kinetics of the unfolding of the apoferritin shell. The experimentally determined values of r, (peak-to-peak distance at 287 and 283nm/peak to peak distance at 295 and 290.5nm) were studied as a function of time and the results of this are shown in Figure 42. From such a study it can be concluded that the unfolding process follows a first-order rate equation and the kinetic parameters $t_{\frac{1}{2}}$ and k were estimated to be 13.8 ± 0.3 min and 2.7 ± 0.2 hr^{-1} respectively. The relatively high value of $t_{\frac{1}{2}}$ as compared to other proteins, the $t_{\frac{1}{2}}$ values for cytochrome C and myoglobin were in the region of 0.5 min, reflects the known stability of the apoferritin protein.

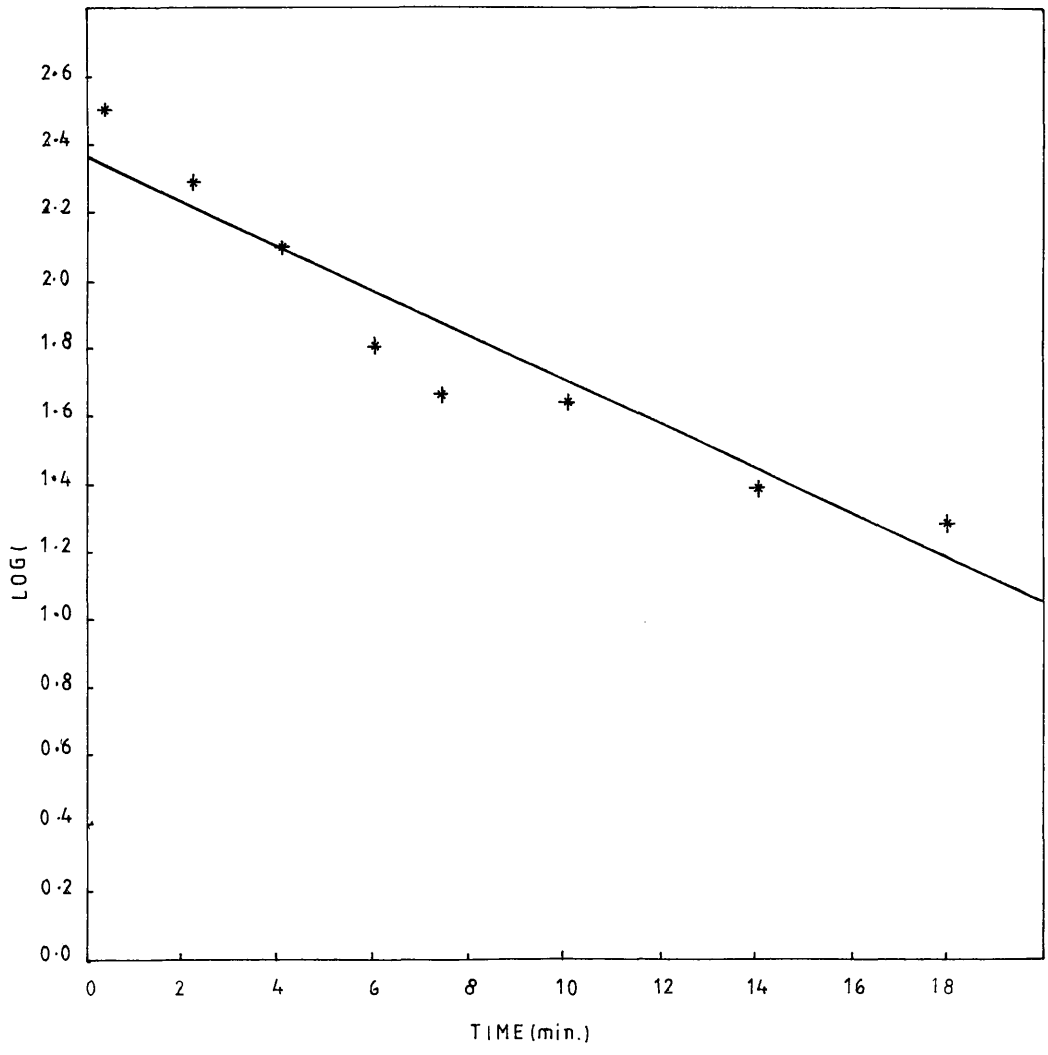


Figure 42: Plot of log (a/ar) vs time to illustrate the first-order nature of the unfolding process.

Nitration of Tyrosine Residues Using Tetranitromethane

Tetranitromethane has been widely used in recent years as a probe for exposed tyrosine residues in a number of proteins. The reaction is carried out under mild conditions and has been found to be specific for tyrosine. The combination of these two effects, specificity and mild reaction conditions has made it the reagent of choice for investigating the importance of tyrosine as a functional group in proteins. The tyrosyl residues may, in some cases, be selectively nitrated in order to assess the effect of each tyrosine in turn on the activity of an enzyme (Cuatrecasas, Fuchs, and Anfinsen, 1968).

In any modification reaction the difference in reactivity of amino acid side chains of a particular kind is due to some side chains being more 'exposed' or 'buried' than others. Groups at the surface may also be masked slightly because of shallow folds caused by neighbouring amino acid residues, resulting in a different reactivity. In addition modification of residues also depends on the size of a reagent and its polarity; small hydrophobic reagents are more likely to be able to penetrate into the protein molecule and to react with groups that are quite a distance from the surface. This has been found to be the case in the nitration of cytochrome C with tetranitromethane as the easily nitratable tyrosine residues were

found, as a result of X-ray crystallography data, to be buried in a hydrophobic environment.

Interaction of tetranitromethane with tyrosine residues introduces a nitro residue into the aromatic ring, *ortho* to the phenolic hydroxyl group, resulting in a 3-nitrotyrosyl derivative as shown below.

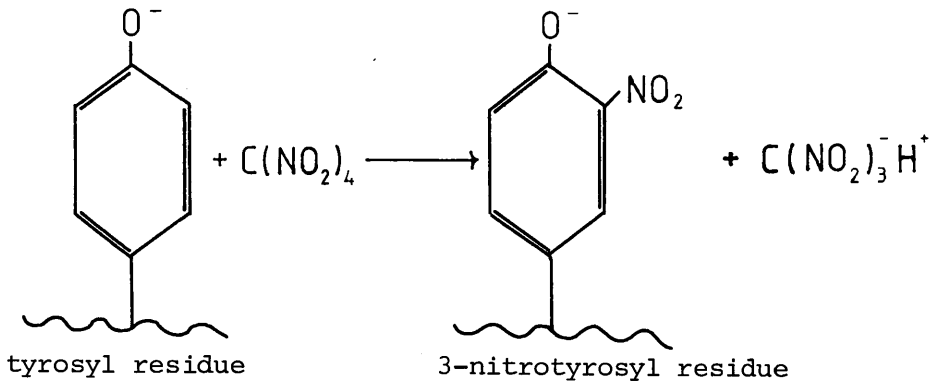


Figure 43: Nitration of tyrosyl residues using tetranitromethane

The extent of modification may be monitored by amino acid analysis or spectrophotometrically. The modified derivative 3-nitrotyrosine is stable to acid hydrolysis (6N HCl, 110°C for 16 hrs) and may be easily identified by amino acid analysis. Using the conventional programme for amino acid analysis this modified residue was found to elute after phenylalanine and before histidine. The degree of modification could therefore be easily quantified from these results, not only by the appearance of 3-nitrotyrosine in the amino acid analysis traces but also by a concomitant decrease in the tyrosine peak.

The 3-nitrotyrosine chromophore absorbs in the visible region of the spectrum with a λmax of 428nm and a molar absorption coefficient of $\varepsilon_{428nm} = 4100$. This provides a convenient and accurate quantitative measure of the extent of reaction. However, determining the extent of nitration spectrophotometrically is complicated because the nitroformate anion produced in the course of the reaction also contributes to the absorbance at 428nm. However, Bustin (1971) has described a method for determining a corrected value for nitrotyrosine from the spectrophotometric data at 428nm. He has shown that the nitroformate anion has a wavelength maximum at 350nm and that the absorption of the anion at 428nm is 4% of the maximum absorption of this species. He further demonstrated that the molar absorption of the nitroformate anion at 350nm is 3.5 times the molar absorbance of nitrotyrosine at 428nm. This being the case we have

$$\text{Absorbance}_{428nm} = \text{Absorbance}^{\text{nitrotyr}}_{428nm} + \text{Absorbance}^{\text{nitroformate}}_{428nm}$$

$$= \text{Absorbance}^{\text{nitrotyr}}_{428nm} + 0.04 \times \text{Absorbance}^{\text{nitroformate}}_{350nm}$$

$$= \text{Absorbance}^{\text{nitrotyr}}_{428nm} + 0.14 \times \text{Absorbance}^{\text{nitrotyr}}_{428nm}$$

$$= 1.14 \times \text{Absorbance}^{\text{nitrotyr}}_{428nm}$$

It can be seen from this calculation that the absorption contribution of the nitrotyrosine residue is 87.8% of the total absorbance at 428nm and not 86% as quoted by Bustin (1971).

Results

The nitration of horse spleen apoferritin was carried out at various temperatures from 11°C to 65°C and the results of these experiments are shown in Figure 44. The stoichiometry of the reaction was calculated using the equation given below, which takes into account the contributing absorbance of the nitroformate ion.

$$\text{Stoichiometry} = \frac{A_{428nm} \times 19,800 \times 0.981 \times 0.878}{4,100 \times A_{280nm}}$$

Where $E^{0.1\%}_{280nm} = 0.981$ for horse spleen apoferritin,

$\varepsilon_{428nm} = 4100$ for 3 nitrotyrosine

and the molecular weight of the subunit was taken to be 19,800.

The modification reaction was allowed to continue for 180 minutes and the end-point of each titration is tabulated overleaf.

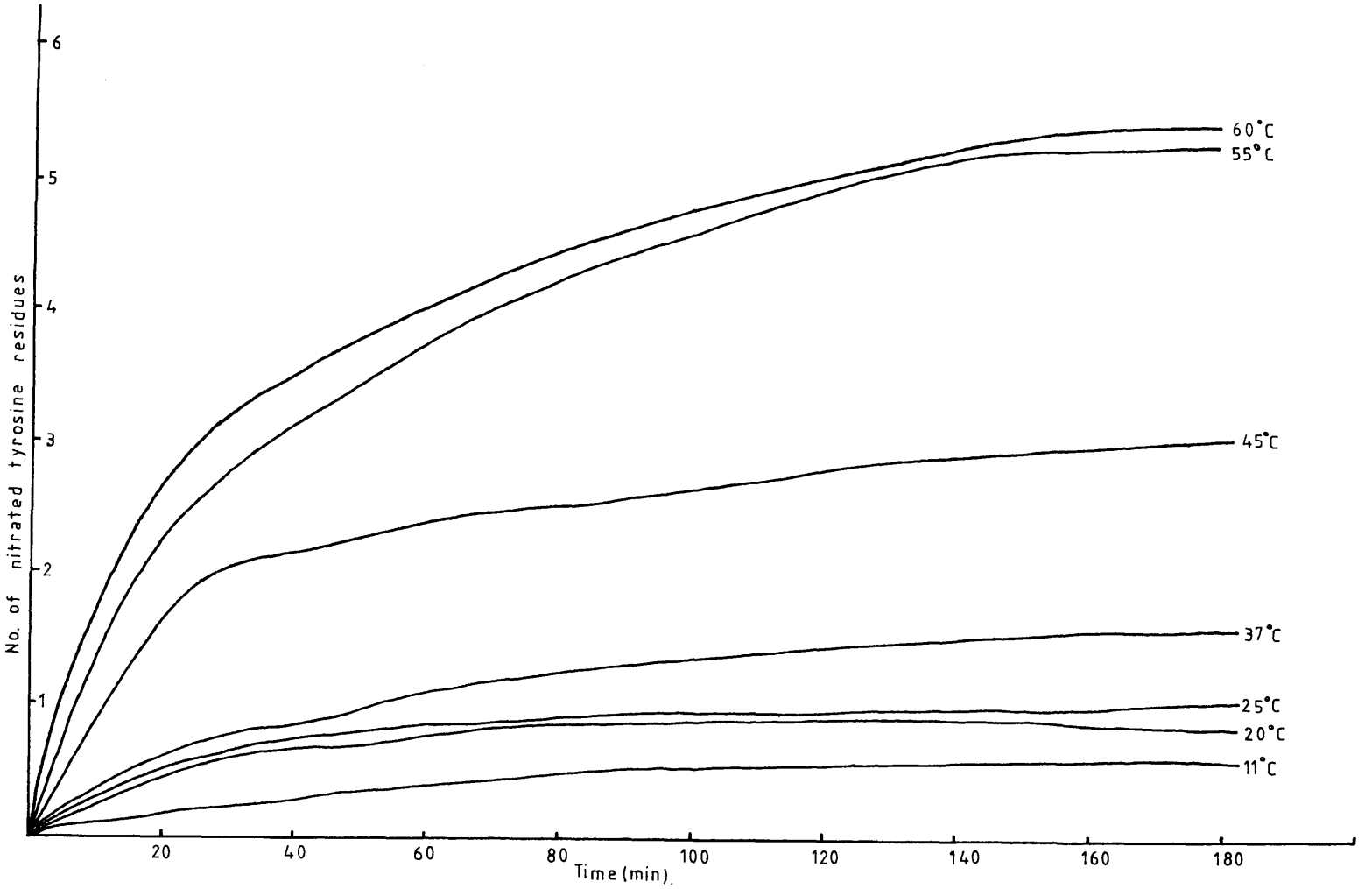


Figure 44: Nitration of horse spleen apoferritin at various temperatures.

Stoichiometry was determined using the equation = $\frac{A_{428nm} \times 19,800 \times 0.981 \times 0.878}{4,100 \times A_{280nm}}$ (See text)

Temp. °C	A_{428nm}	A_{280nm}	NO_2-tyr/subunit
11	0.142	0.981	0.60
20	0.204	0.960	0.88
25	0.277	1.094	1.05
37	0.384	0.959	1.67
45	0.674	0.912	3.07
55	1.222	0.968	5.25
60	1.348	0.974	5.75

Table 8: End-point of nitration using tetranitromethane

As one would expect the number of tyrosine residues nitrated per subunit increases with temperature. This observation is probably a result of the increased kinetic energy of the reactants together with a conformational change of the protein at elevated temperatures. This loss of conformation probably results in more tyrosine residues being orientated towards the surface of the apoferritin molecule. Also the opening of a usually compact structure enabling the reagent to react with previously inaccessible tyrosine residues may be a contributing factor.

At temperatures of 11, 20 and 25° C the number of tyrosine residues nitrated per subunit were 0.60, 0.88 and 1.05 respectively. At these temperatures the

titration was essentially over after 60 minutes, with little change occurring thereafter. This indicates that only slight, if any, changes in the conformation of the protein occur at these temperatures.

The results of the titration at 37°C shows a similar profile to those at the lower temperatures with the end-point being 1.67 nitrotyrosines per subunit. This increase in nitratable tyrosines may be due to the tyrosine residue which is present in helix E being partly exposed to solvent or possible alteration in the conformation of helix A where four tyrosine residues are located. However at 37°C one would only expect very minor conformational changes to occur.

At 55 and 60°C, 5.25 and 5.75 tyrosyl groups per subunit were nitrated respectively indicating that disruption of the tertiary and quaternary structure of the molecule has occurred. These values agree with the results obtained by second derivative spectrophotometric titrations (see page 156) and also the six reported tyrosine residues in the horse spleen apoferritin sequence of Heusterpreute and Crichton (1981). Silk and Breslow (1976), in examining tyrosine ionization as a function of pH, found that ionization only occurred above pH 11 and that loss of conformation was a pre-requisite for the complete titration of all tyrosine residues.

The results of the reactions carried out at low temperatures suggest that only one tyrosine residue per subunit is nitrated which is in agreement with the data obtained by Crichton and Bryce, 1973. However it may be that the nitration of several residues has occurred to give a composite value of one nitrotyrosine per subunit. As indicated earlier the reactivity of tyrosine residues reflects not only their degree of exposure to solvent but also their local environment and this may be the case with horse spleen apoferritin. One approach to establishing which of the above cases is correct would be to digest the nitrated protein with, for example, trypsin and then after isolating the peptide fragments examine them by amino acid analysis and Edman degradation.

When one examines the electron density maps of horse spleen apoferritin in conjunction with the recently reported sequence, the location of the tyrosine residues may be determined. The six tyrosine residues occur at positions 8, 23, 28, 30, 36 and 164. The tyrosine at position 8 occurs in the non-helical N-terminal region of the protein whereas the remaining tyrosines are found within helices. Those tyrosines at positions 23, 28, 30 and 36 all occur in helix A and the last tyrosyl residue at sequence No.164 is located in the short helix E which lies perpendicular to the four main helices. It would therefore seem likely that the tyrosine which

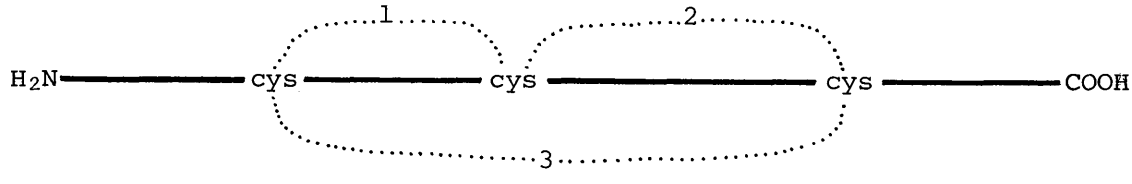
occurs in the N-terminal region is accessible to solvent and that it is this tyrosine residue which is nitrated at low temperatures. The remaining tyrosine residues are thought to be buried within the subunit or occur at its subunit-subunit interfaces (Bourne *et al*, 1982a).

At low temperatures the nitration was essentially complete after 60 minutes. In contrast the reactions which were carried out at higher temperatures showed a gradual increase in the value of the nitrated tyrosine residues throughout the time course reflecting a gradual loss of conformation. The fact that this change occurs slowly over the entire 180 minutes is another indication of the stability of this protein.

Cleavage at Cysteinyl and Cystinyl Residues using 2-nitro-5-thiocyano benzoic acid

When horse spleen apoferritin is analyzed by electrophoresis on SDS gradient-pore polyacrylamide gels in both the absence and presence of thiol reagents it can be shown that disulphide bonds are present in the molecule (see earlier section). The additional protein bands which occur in the gels in the absence of 2-mercaptoethanol are thought to represent intramolecular disulphide bonds within the subunit and inter-molecular disulphide bonds occurring between two subunits. The addition of thiol to these preparations results in a much simpler band pattern within the gel and a much more heavily stained subunit band. These results have been confirmed by a two dimensional diagonal electrophoretic technique which clearly shows that both inter- and intra-molecular disulphide bonds occur in the presence of SDS.

The cysteinyl content of horse spleen apoferritin has been reported to be 2-3 residues per subunit (Crichton *et al*, 1973). If three cysteinyl residues are present then there are three possible ways in which a single disulphide bond may be formed in the subunit.



The remaining free cysteinyl residue would be available for the formation of intermolecular disulphide bonds with other subunits. If however only two cysteines are present then they may both be involved in a disulphide bond within the subunit or may take part in intermolecular disulphide bond formation.

The present study was undertaken to provide further data on the cysteinyl content of horse spleen apoferritin subunits. The subunits were cleaved at cysteine using the reagent 2-nitro-5-thiocyanobenzoic acid. It was thought that this approach would not only determine the number of cysteine residues in the subunit but also provide further information on their location within the sequence.

The reagent 2-nitro-5-thiocyanobenzoic acid was introduced by Degani and Patchornik as a reagent for the selective cyanylation of thiol groups under very mild conditions. Later Jacobson *et al* (1973) described a protocol for the quantitative cleavage of proteins at the amino side of cysteinyl and cystinyl residues using this reagent. The reaction, which involves two stages, results in the formation of an amino terminal peptide and a 2-iminothiazolidine-4-carboxylyl C-terminal fragment.

In the initial stage of the reaction cysteine and cystine residues are quantitatively converted to

S-cyanocysteine using the reagent 2-nitro-5-thiocyanobenzoic acid. Cleavage of the protein is the brought about by increasing the pH where a base-catalysed reaction results in the formation of a 2-iminothiazolidine-4-carboxylyl peptide.

The proposed mechanism of this second stage of the reaction is outlined below:

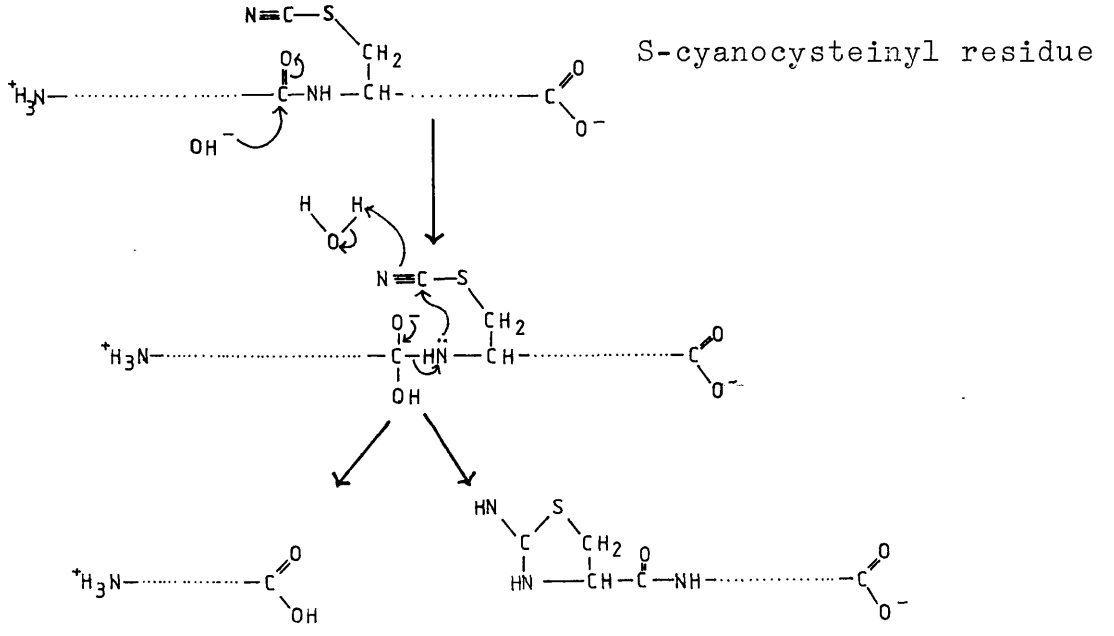


Figure 45: Proposed cleavage mechanism at cysteinyl residues using 2-nitro-5-thiocyanobenzoic acid.

Results

The material obtained after chemical digestion was applied to a Sephadex G-100 column equilibrated with 6M guanidine hydrochloride, 0.04M potassium phosphate pH 7.0, to separate the peptides resulting from this cleavage mechanism. The results of this column chromatography are shown in Figure 46. When material from each of the three peaks was analyzed by SDS electrophoresis, peak I was found to contain mostly material which had not been digested although some minor bands were visible. SDS gel electrophoresis of the remaining fractions II and III both gave a similar band pattern indicating the presence of two peptide fragments (see Figure 47).

Discussion

The presence of only two cleavage bands is not consistent with either two or three cysteinyl residues being present per subunit as one would expect either three or four peptides to have been formed. However since the recent publication of the sequence of horse spleen apoferritin the location of the cysteinyl residues is now known. Only two cysteines are reported to be present at positions 48 and 126.

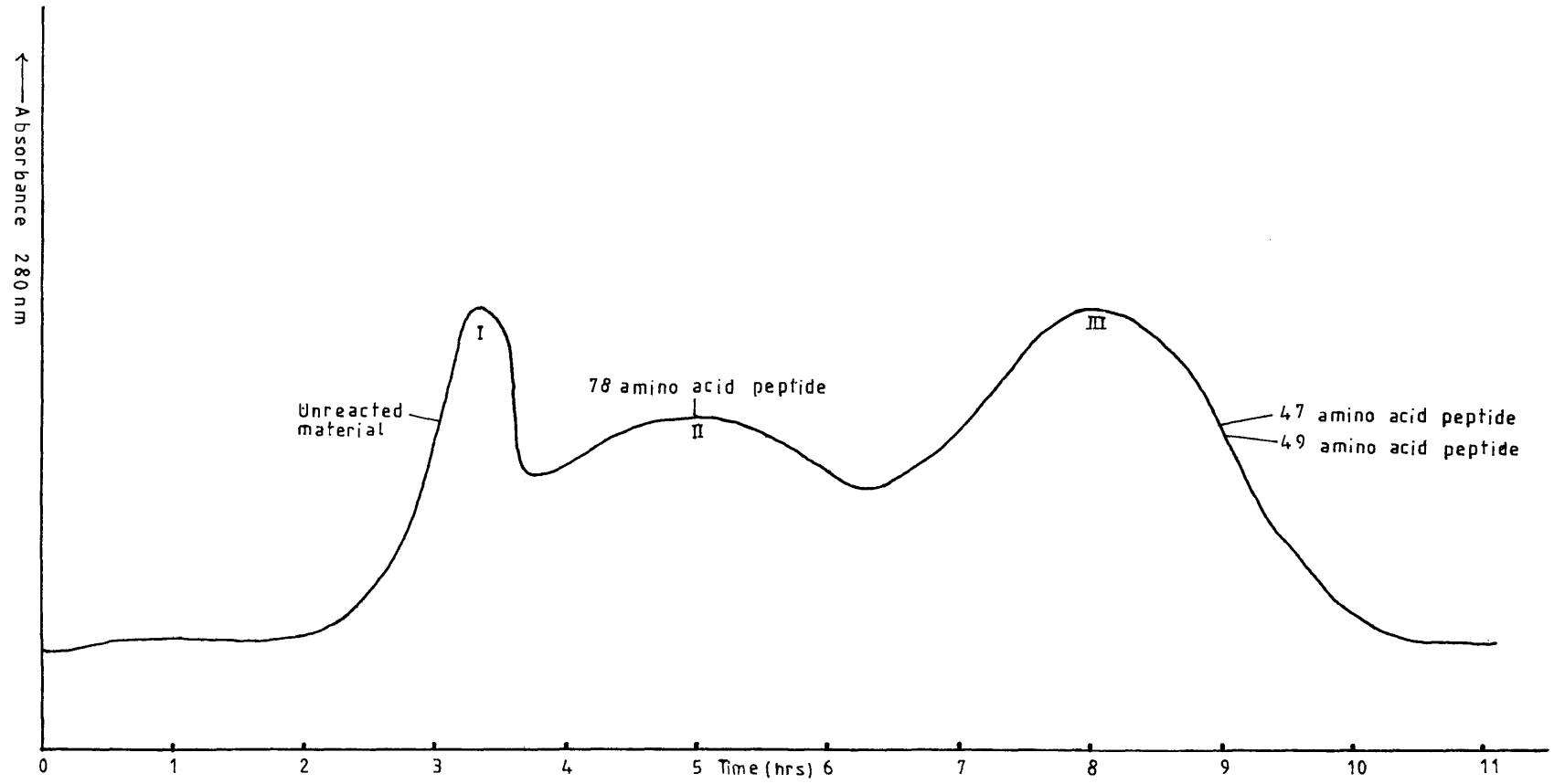


Figure 46: Separation of chemically digested horse spleen apoferritin subunits using 2-nitro-5-thiocyanobenzoic acid.

Sephadex G-100 column equilibrated with 6M guanidine hydrochloride, 0.04M potassium phosphate, pH 7.0
Flow rate 9 ml/hr.

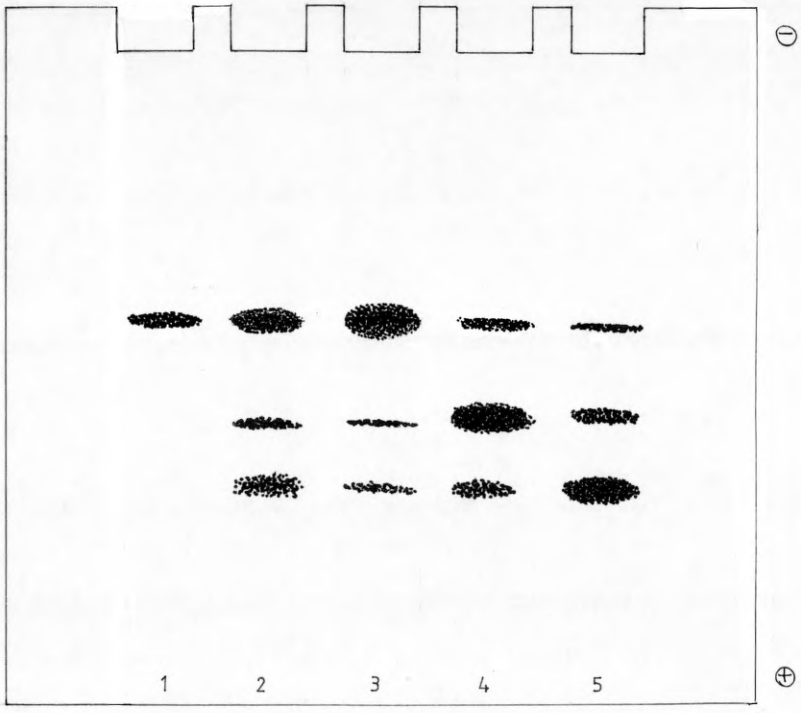
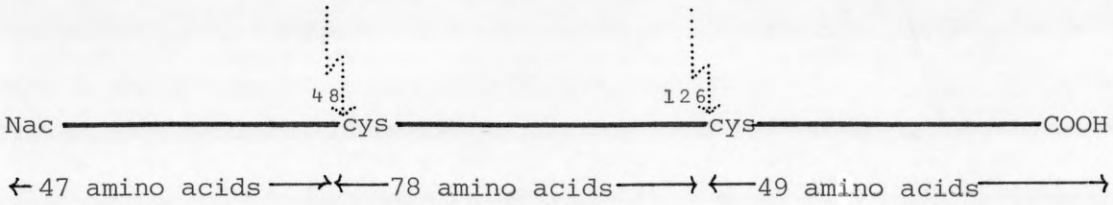


Figure 47: Schematic representation of horse spleen apoferritin subunits cleaved at cysteine residues and electrophoresed on SDS polyacrylamide gels.

Lane 1 Horse spleen apoferritin
Lane 2 Chemically digested horse spleen apoferritin
Lane 3 Peak I, G—100 column (see figure 46)
Lane 4 Peak II "
Lane 5 Peak III "



When one examines the results of this cleavage mechanism in the light of the sequence data then the presence of only two cleavage products as judged by analysis using SDS gel electrophoresis can be expected. Two of the resulting peptides differ in size by only two amino acids. It is therefore unlikely that these two peptides would be resolved by either column chromatography in 6M guanidine hydrochloride or SDS polyacrylamide gel electrophoresis as molecular weight is the basis of separation in both cases. However by utilizing a property which depends on the amino acid composition, such as surface charge, one would expect these two peptides to be separated by techniques such as ion-exchange chromatography or preparative hplc.

N-terminal determination of material from peaks I, II and III were carried out using the dansyl chloride method of Grey (1972). Dansyl chloride is reactive towards a variety of bases, the most important of these are the primary and secondary amines. It is susceptible to hydrolysis by water and hydroxyl ions, however coupling to the α-amino group can only take place in alkaline pH so that labelling is always in competition with hydrolysis.

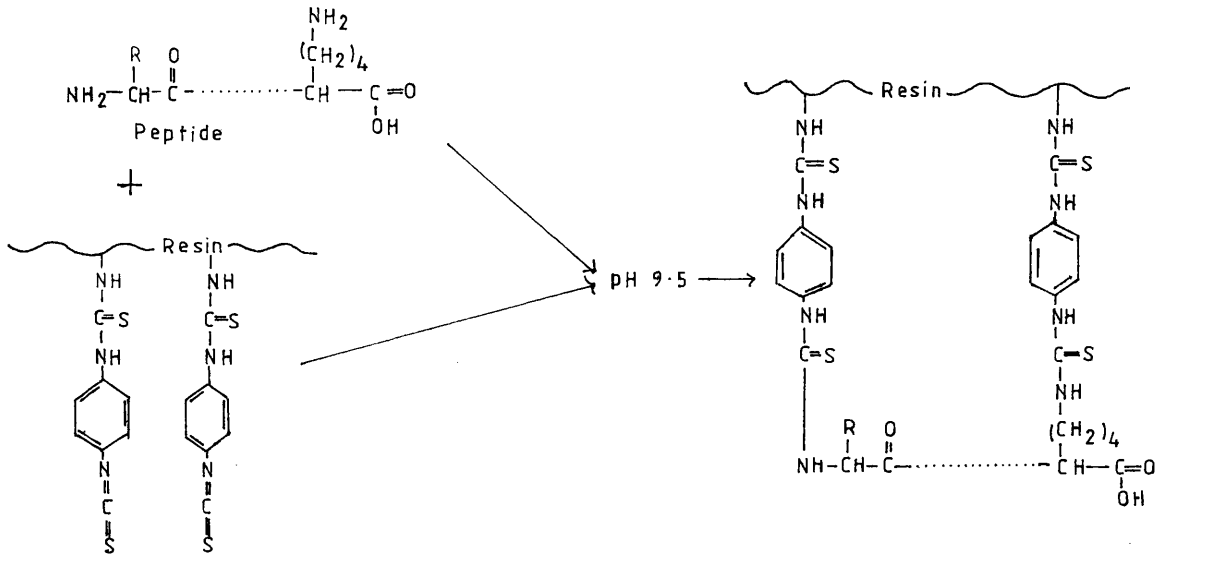
For this reason an excess of reagent was used during labelling. This method proved unsuccessful since the N-terminal amino acid of horse spleen apoferritin is acetylated and the position to which DNS-cys migrates is masked by DNS-OH and these two products could not be resolved by the various solvent systems used.

Sequencing Studies

Sequencing studies were carried out using an LKB 4030 solid-phase sequencer. In the solid-phase method of protein sequencing the material to be sequenced is attached to an inert support through either the C-terminal carboxyl group or through a side-chain function. A series of reactions is then carried out which follows the Edman degradation procedure as outlined in Figure 48.

The Edman degradation is a two-step process. The first stage involves the coupling of phenylisothiocyanate to the α-amino group of a peptide or protein to produce a phenylthiocarbamyl derivative. This initial stage of the process must be carried out under alkaline conditions pH 8-9, as the α-amino group must be unprotonated for thr reaction to occur. After removal of excess coupling reagent the phenylthiocarbamyl peptide is treated with anhydrous trifluoroacetic acid which causes the cyclization and immediate release of the N-terminal amino acid as an anilinothiazolinone derivative. Since these derivatives are unstable (especially those of serine and threonine) they are converted to stable phenylthiohydantoin (PTH) derivatives before identification.

The choice of support in solid-phase sequencing depends on the size of the protein or peptide to be sequenced. In general, polystyrene based supports are



Attachment of peptides using phenylenediisothiocyanate.

Edman degradation is then performed as follows:

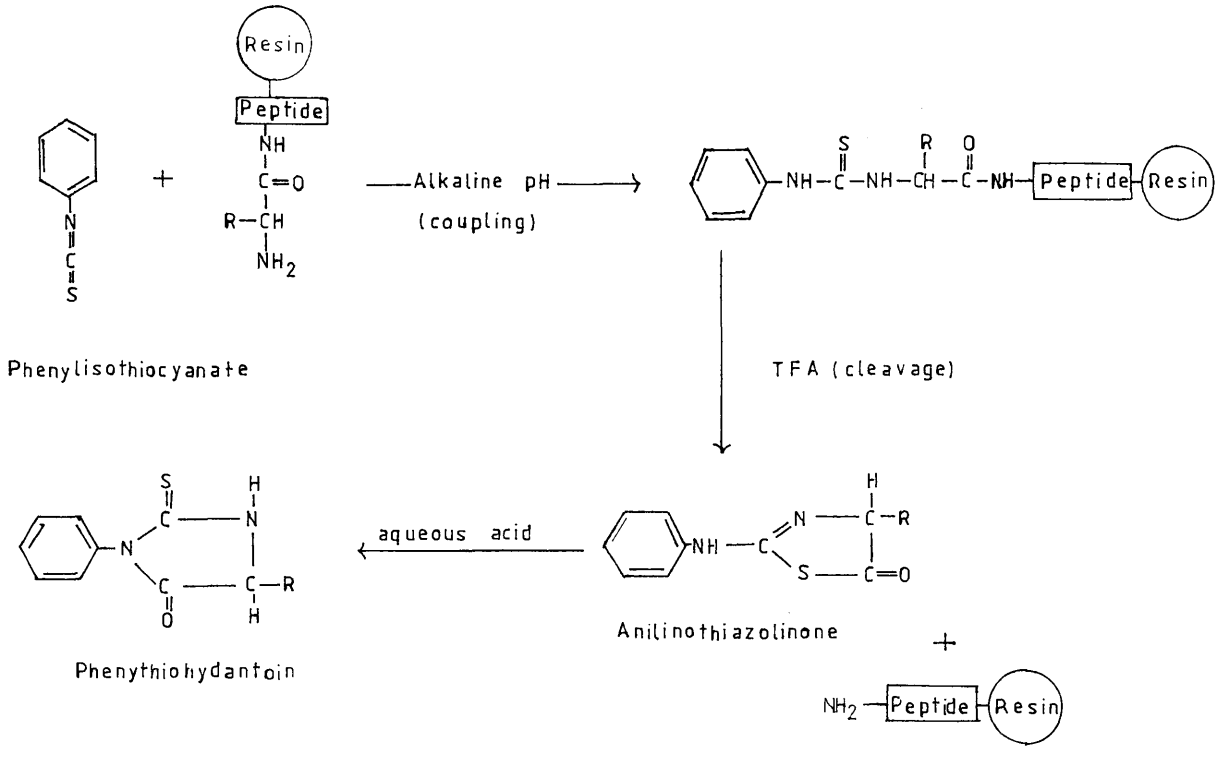


Figure 48: The Edman degradation

used for smaller peptides whereas the more porous glass supports are used for larger peptides and proteins. In this study proteins and peptides greater than 20 amino acids were normally sequenced and a glass support, 3-aminopropyl glass, was chosen as the support material.

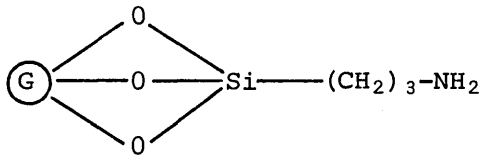


Figure 49: 3-aminopropyl glass support (G) ≡ glass

The proteins and peptides were attached to the support using p-phenylenediisothiocyanate (DITC). This reagent forms cross-linkages between the amino groups present on the support and any amino groups present on the peptide, namely the α-amino group and ε-amino groups of lysine. The reaction procedures are summarized in Figure 48 When the first cycle of Edman degradation is carried out the N-terminal amino acid is cleaved from the rest of the peptide leaving a free amino group. The identity of this first amino acid cannot be determined as it is retained on the support. In subsequent cycles the anilinothiazolinones are released from the support and can be identified by hplc, tlc and back hydrolysis to the parent amino acid except in the case of lysine where the ε-amino group causes it to be retained on the support. Any lysine residue therefore appears as a gap in the sequence.

The peptides and proteins used in this study were attached to an aminopropyl glass support which had been previously activated using phenylenediisothiocyanate.

To determine the efficiency of this coupling procedure a small sample (∿5 mg) was taken and, after hydrolysis, analyzed on an amino acid analyzer. If this proved satisfactory the protein or peptide was sequenced using a standard single column sequencing programme as described in the Materials and Methods section. A commercially prepared sample of myoglobin attached to aminopropyl glass was also sequenced at regular intervals using this programme to determine the performance of the sequencer and also the prepared solvents. This also gave an indication of the extent of background and overlap that could be expected using this system.

Protein and peptide sequencing has been used to determine the results of chemical modification and cleavage procedures. The results of this sequencing are described elsewhere in the text.

Many tissue ferritins contain multiple forms of ferritin, isoferritins, which appear to differ structurally and immunologically. The isoferritins are thought to represent varying proportions of the two subunits, H and L, forming a 24 subunit molecule. The two polypeptide chains share extensive sequence homologies as judged by peptide mapping (Arosio, Adelman and Drysdale, 1978) but it is not clear whether they are derived from distinct mRNA sequences or by post-translational modification. The determination of the amino acid sequence of these two polypeptides should permit the resolution of a long

standing controversy over their origin and function in the assembled 24-mer.

In this study an attempt was made to sequence the N-terminal region of the heavier molecular weight subunit, H. It was thought that if after the first ∿20 amino acids the sequence starts to correlate with the known sequence of the L subunit then this would indicate that H is the precursor of L.

Using an LKB Uniphor a preparative gradient-pore polyacrylamide SDS gel was prepared, and a sample of horse spleen apoferritin was electrophoresed. Fractions from this gel were then taken and analyzed on similar analytical SDS gels to determine their purity. This procedure enabled a sample of pure L subunits to be obtained. This protein was covalently attached to p-phenylenediisothiocyanate activated amino propyl glass and sequenced on an LKB 4030 sequencer. Amino acid analysis showed that the protein had coupled to the glass support. However, due to the presence of an N-terminal acetyl group the α-amino group was unable to react with phenylisothiocyanate hence no release of the N-terminal amino acid occurred on treatment with trifluoroacetic acid. This result implies that if a sample of 'H'-rich subunits was obtained and coupled to the support then any L-subunits which were present would be unable to be sequenced and therefore allow the sequence of the heavier subunit to be obtained.

Using a similar system a sample of 'H'-rich subunits was obtained (∿60% H, 40% L) and attached to amino propyl glass for sequencing. As with the preparation of L-subunits no sequential degradation took place. This would indicate that the 'H'-subunits are also blocked at the N-terminal end of the polypeptide. Due to insufficient time to complete this part of the study it was not possible to carry this experiment any further. If a sample of pure H subunits was prepared and the N-acetyl group removed, allowing sequencing to take place, then it may be possible to shed some light on the controversy over H and L subunits.

Although circumstantial, the results obtained would suggest that L and H are separate entities as it is unlikely that nature would expend energy on acetylating a precursor molecule if that portion of the molecule were not to be used in the final product.

The sequence of human spleen apoferritin has recently been published by Wustefeld and Crichton (1982) enabling the sequences of horse spleen and human spleen to be compared. The two sequences are very similar with only 14% difference between them and most of the changes being conservative. Interestingly these workers found small amounts of peptides which they were not able to fit into the sequence but which did show homology to the main sequence. They have concluded that, as their

preparation of human spleen apoferritin contained ∿10-20% H subunit these sequences belong to a minor component namely the H subunit.

Studies on the Synthesis of Ferritin in Morris Hepatoma Cells

The experiments described in this section were undertaken whilst on a two month visit to Professor I. Listowsky's laboratories at the Albert Einstein College of Medicine, Bronx, New York.

The aim of this study was to investigate the effect of the addition of iron to the growth medium on a Morris hepatoma cell line. The way in which the cells responded to the supplement of iron was examined from two aspects; firstly to assess the quantity of ferritin produced by these cells and secondly to determine if increasing the iron concentration caused any change in the subunit composition of nascent ferritin. It was thought that increasing the iron concentration of the surrounding medium and therefore within the cell would stimulate the production of ferritin subunits and that one subunit (e.g. H or L) may be preferentially synthesized.

The normal procedure by which a cell is able to obtain iron is via the extracellular protein, transferrin. This molecule which is present in the circulatory system is capable of binding two ferrous ions which the cell is able to utilize. Once iron enters the cell it may be directed towards the synthesis of iron-containing enzymes, cytochromes or incorporated into the iron-storage protein, ferritin. However, little is known

about the mechanism by which iron enters the cell and is then transported within the cytosol, nor is any detailed mechanism known of how iron is able to accelerate the synthesis of ferritin. Some workers (Zaman and Verwilgen, 1981; Jacobs, 1977; White and Jacobs, 1978) have found evidence for the existence of a non-haem, non-ferritin iron pool within the cell. It has been suggested that newly accumulated iron first goes into an iron pool whence it can be used for other cellular processes including deposition into ferritin.

Recent evidence suggests that the degree of iron-loading within the cell may result in a change in the isoferritin pattern. Hoy and Jacobs (1981) in their investigation on Chang liver cells found that a shift towards more acidic ferritins occurred on iron loading. However this change in the isoferritin pattern was not solely dependent on the net proportion of H and L subunits as these workers found that at a given pI iron loading increased the proportion of ferritin reacting to heart antibody. This would indicate that changes in the immunoreactivity induced by iron is not solely dependent on subunit composition alone. Indirect evidence which supports the view that such a change in the isoferritin pattern does occur on iron-loading has come from Wagstaff, Worwood and Jacobs (1978). They found that H-rich ferritins, for example heart ferritins, are more efficient at oxidizing iron than the more basic

L-rich forms. This gives an indication of the physiological purpose of an isoferritin shift on iron loading.

In this investigation cell culture was used as it provides a valuable model for studying the intracellular uptake of iron by a cell and also the cellular responses which occur on increasing the iron content of the cells surrounding environment.

The first experiment investigated the way in which the cells were affected by the addition of iron to their growth medium in terms of the amount of ferritin synthesized. The quantity of ferritin produced was determined by competitive inhibition assays and a 2-site radioimmunoassay. The second experiment examined the incorporation of ^{14}C-leucine into the cells when incubated in iron-supplemented media as in experiment one. This was to determine if any changes in the newly synthesized ferritin could be detected on iron-loading. The labelled material was precipitated immunochemically and then subjected to sodium dodecyl sulphate electrophorsesis with fluorimetry to determine if any change in the distribution of H and L subunits occurred which could be related to the degree of iron-loading.

Results

Iron Overloading Studies

Cell Growth

Figure 50 shows a series of growth curves of the Morris hepatoma cell line $H_4A_ZC_2$ cultured with and without iron supplemented media containing 10% foetal calf serum. The cultures were maintained in RPMI 1640 media with iron sources of ferric ammonium citrate (Figure 50a) and ferricnitrilo^{tri}acetate (Figure 50b) at concentrations of 50µmol, 100µmol and 200µmol ferric ion. The responses of these cells to either iron donor was a stimulation of ferritin synthesis.

Initial experiments showed that growth of the hepatoma cell line was retarded at iron concentrations of 250µmol. Exposure of the cells to iron concentrations of 500µmol as ferricnitrilo^{tri}acetate caused cell death however cells incubated with the same concentration of ferric ammonium citrate remained viable.

The hepatoma cells were seeded at 2.6×10^5 cells per 60mm petri dish and 3.5×10^6 cells per dish were counted on day 0, the first day of the experiment. The control cells (without iron) increased in number exponentially up to day 3 and then levelled off at $\sim 4.5 \times 10^6$ cells per dish. Cells grown in iron-supplemented media showed similar growth curves to the control cells although their

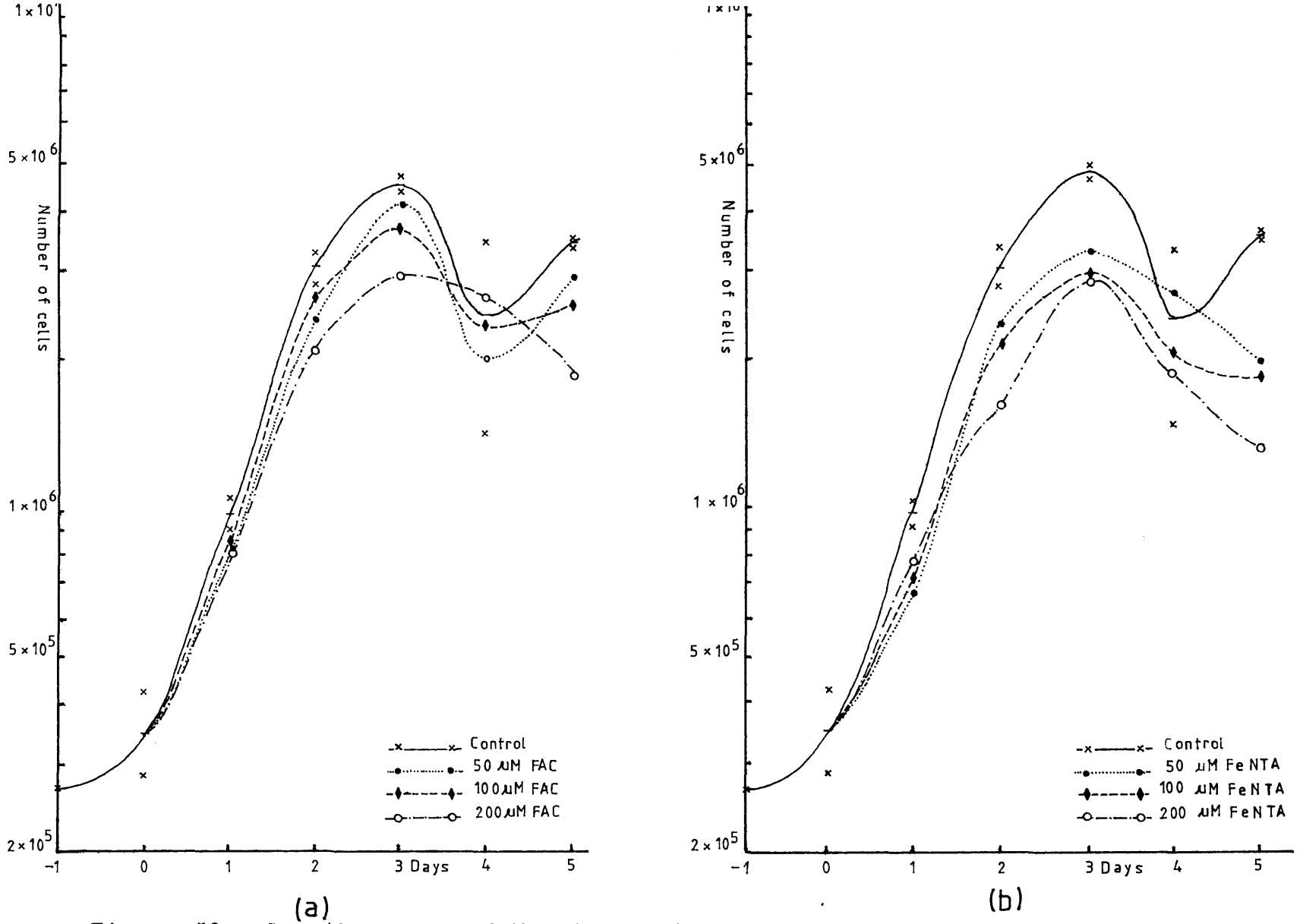


Figure 50: Growth curves of Morris hepatoma cell line $H_4A_zC_2$ cultured with and without iron supplemented media.

a) ferric ammonium citrate
b) ferricnitriloacetate.

numbers suggest a slight inhibition of growth in high concentrations of iron donor. After 3 days cell detachment from the dishes was accelerated, this was especially noticeable in media supplemented with ferricnitriloacetate.

Ferritin levels in cell extracts

Figures 51a, 51b, show the change in ferritin concentration in cell extracts harvested at 24 hour intervals in parallel with cell growth studies (Figure 51 c, 51 d). The ferritin concentration was determined by a two-site radioimmunoassay. The working range of the assay was between 15 ng and 1000 ng/ml. A competitive assay procedure was also used initially however the 2-site radioimmunoassay proved to be far superior in terms of both reproducibility and the working range of the assay.

The response of Morris hepatoma cells to either iron donor was a stimulation of ferritin synthesis. As can be seen from the graphs (Figures 51 a, b) the control cells produced low levels of ferritin. On days 0 and 1 the level of ferritin in these cells was too low to be detectable but rose to 260 ng/10^6 cells on day 3. Trace amounts of iron and/or the presence of bovine transferrin in the foetal calf serum may partially account for this production of ferritin which may however be considered as near background levels.

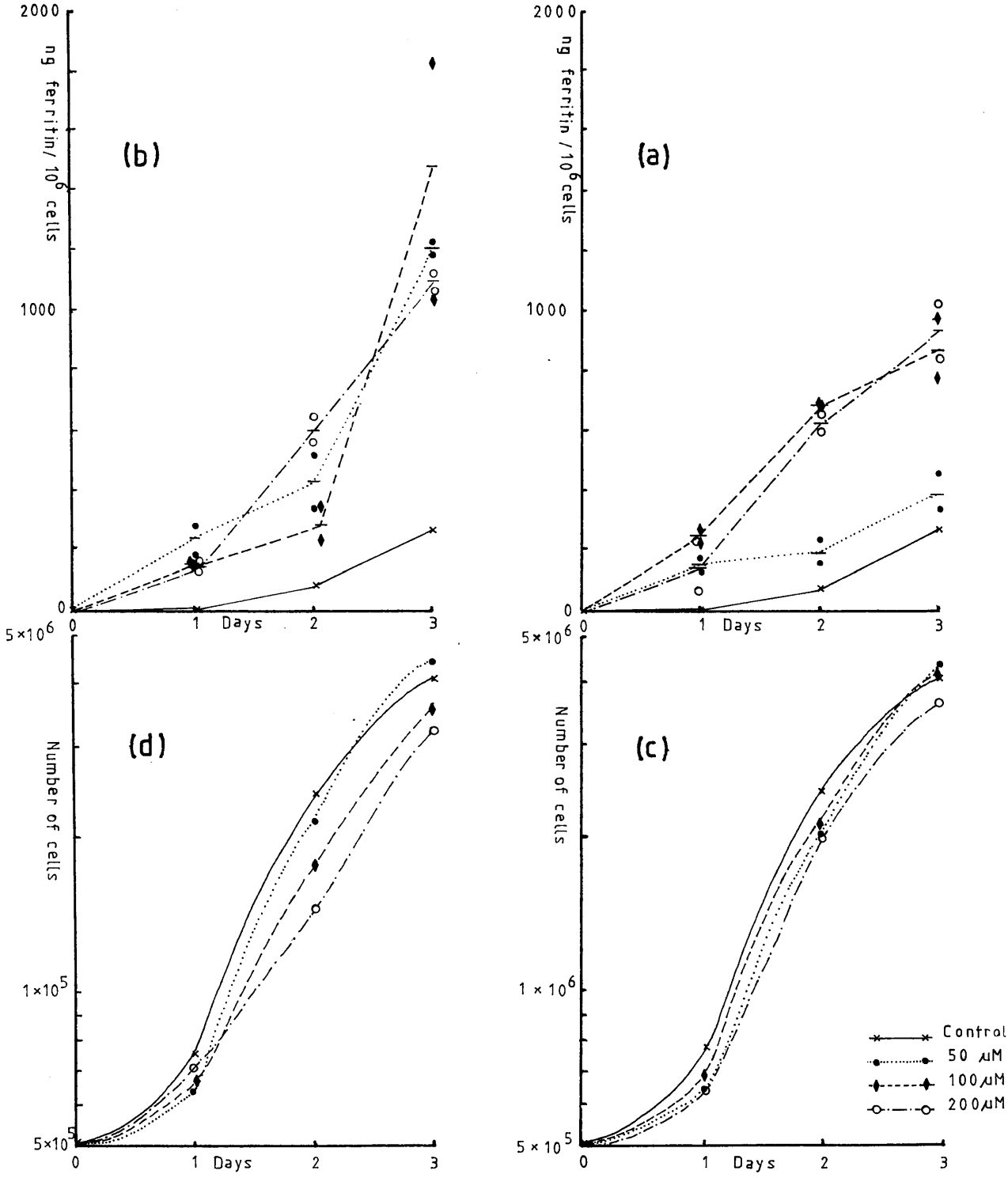


Figure 51: Ferritin levels in Morris hepatoma cell line and corresponding growth curve.

Cells were harvested at 24 hour intervals and ferritin concentration determined by 2-site radioimmunoassay

a) Ferritin levels in cells incubated in ferric ammonium citrate
b) Ferritin levels in cells incubated in ferric nitrilotriacetate
c) Cell growth curve, ferric ammonium citrate
d) Cell growth curve, ferric nitrilotriacetate

Cells incubated with ferric ammonium citrate showed a rapid rise in cytosolic ferritin at iron concentrations of 100 and 200µmol over the three day period. On day 3 the ferritin concentrations were 880 ng and 930 mg per million cells respectively and both these values were more than 3-fold greater than the control. The cells incubated at 50µmol iron showed a slower accumulation of ferritin but this was still elevated above the control. The concentration of ferritin on day 3 was 400 ng/10^6 cells an increase of 1.5 fold over the control cells.

Induction of ferritin synthesis was greater with ferric nitrilo^{tri}acetate than with ferric ammonium citrate. This may be the result of a protective mechanism of the cell against the toxicity of ferric nitrilo^{tri}acetate. On day 3 the ferritin concentration of cells incubated at 50, 100 and 200µmol iron were 1200, 1480 and 1320 ng per million cells respectively. Iron concentrations of 100µmol appeared to be optimal, and on day 3 the level of ferritin in cells incubated at this concentration was nearly five fold greater than the control.

Very low levels of ferritin were detected in the media, normally values of 30 ng/ml or less were obtained throughout the period of the experiment.

Study of ^{14}C-leucine uptake by hepatoma cells

The Morris hepatoma cells were seeded onto 30 mm petri dishes and incubated in RPMI 1640 media containing 10% foetal calf serum until they had reached confluency. At this stage the synthesis of cellular proteins is at a 'turn over' level allowing the cell to direct its energy into synthesizing other proteins as demanded by its environment. At this time the media was replaced with 2.0 ml iron conditioned media containing 25, 50, 100 and 200μM of either iron donor. 4μCi ^{14}C-(DL) leucine (specific activity 54 mCi/mmol) were added to each dish. After 24 hrs the cells were washed and harvested. The newly synthesized ferritin from the cell lysates was then precipitated immunochemically. These pellets were solubilized in SDS buffer and applied to gradient pore SDS polyacrylamide gels. After electrophoresis the radioactivity was visualized by fluorography. In the initial experiments insufficient counts were obtained in the immunoprecipitates to detect any nascent proteins visually by fluorimetry although ferritin subunit bands were detectable by conventional staining techniques using Coomassie Blue. It was hoped to repeat these experiments using RPMI 1640 media devoid of leucine so that only labelled leucine would be present and also increase the number of cells. Unfortunately due to the short duration of this visit there was insufficient time to carry this out before returning to Scotland.

Discussion

The metabolic functions of iron are diverse and involve all the tissues of the body. Although a considerable amount of information is available regarding the role of iron in haem compounds and non haem proteins our knowledge of cellular iron metabolism in pure populations of living cells is scanty. Early work carried out by Richter (1961) showed that the addition of small quantities of ferrous sulphate to the medium of HeLa cells activated the synthesis of the ferritin molecule. Since that time the stimulation of ferritin synthesis has been shown to occur in several cell lines including Chang liver cells (Bailey-Wood, White and Jacobs, 1975) rat hepatocytes and Kupffer cells (Doolittle and Richter, 1981) and now the Morris hepatoma cell line $H_4A_ZC_2$. These cell lines provide valuable systems in which several aspects of cellular iron metabolism can be studied including the uptake and subsequent intracellular distribution of iron as well as ferritin synthesis.

White and Jacobs (1978) used Chang liver cells to examine the possibility of inducing iron overload in these cells. Using three types of donor - transferrin, ferric nitrilotriacetate and ferric citrate they looked at the intracellular distribution of labelled ^{59} Fe iron. They found that most of the labelled iron from transferrin was found in membrane-free supernatant and that 50% of this was in the form of ferritin. When iron was present

as ^{59} Fe ferricnitriloacetate the incorporation of iron was 30-fold greater than saturated transferrin but in this case most of the iron was membrane bound. Of the smaller proportion of iron found in the cell sap, again about half could be accounted for as ferritin. The total uptake from ferric citrate was quantitatively comparable to transferrin although less iron was found in the cell sap. This implies that cellular incorporation of iron from the nitrilotriacetate and citrate complexes, in the absences of transferrin, occurs by the presence of an alternative mechanism to that involving transferrin receptor sites. White and Jacobs (1978) showed that removal of iron from ferritin, by chelation experiments with desferroxamine, could occur with equal mobility whether initially labelled with iron from transferrin, nitriloacetate or citrate complexes. They concluded that ferric nitrilotriacetate and citrate complexes provided a suitable cell culture model of iron loading.

In this study on Morris hepatoma cells the effect of iron loading in terms of ferritin synthesis was examined. The results clearly show that iron acts as a stimulus for the synthesis of ferritin subunit proteins. This is true whether the iron source is from ferricnitrilotriacetate or ferric ammonium citrate. As with Chang cells (White and Jacobs, 1978) the hepatoma cells responded more rapidly to ferricnitrilotriacetate than with

ferric ammonium citrate and the amount of ferritin synthesized was also greater. This may be due in part to a different mechanism by which cellular incorporation of iron occurs as the negative charge of ferricnitrilotriacetate may facilitate penetration of the cell membrane. Once iron has entered the cell the ferric ion must be released from these complexes in order to enter the internal cavity of the ferritin molecule, and the ease by which these ferric ions are released may be related to its ability to trigger ferritin synthesis.

In this investigation the amount of ferritin in the cytosol was quantified and shown to increase in relation to the amount of iron present in the media. It would be interesting to extend this study using ^{59}Fe labelled iron donors and also transferrin to determine the destination of iron once it had entered the cell. This would provide more information on the mechanism of iron uptake and whether it was located within ferritin, an intracellular iron pool or in the membrane.

When Chang liver cells are grown in iron rich media over 20 weeks upto 10 times the normal cellular ferritin levels may be obtained. Hoy and Jacobs (1981) found that over this period a consistent shift to more acidic ferritins occurred when cells were incubated with ferric nitrilotriacetate. In addition to the appearance of more acidic isoferritins (as determined by chloride elution on DEAE cellulose) there was a change in immunoreactivity.

This was shown by an increase in isoferritins reacting to heart antibody. These observations are consistent with the view that the more acidic H subunits are synthesized preferentially in the presence of iron. However these workers found that at a given pI (and therefore one would assume fixed subunit composition) there was an increased proportion of ferritin reacting to heart antibodies. This would suggest that changes in immunoreactivity induced by iron are not entirely dependent on subunit composition alone.

At present the formation of apoferritin by the assembly of two different gene products has not been conclusively demonstrated and the contrary view is that some post translational modification occurs either before or after they are assembled into ferritin. The approach of this study was to examine the problem from a different angle and look at newly synthesized ferritin which had been induced by the presence of iron. It was hoped that this would lead to information on whether changes in the subunit composition of ferritin occurred resulting from one subunit being preferentially synthesized. Unfortunately the initial results from this study are inconclusive due to lack of time but an approach of this type may be able to provide some valuable information and further investigations should be carried out.

It may be that when ferritin molecules contain their full complement of iron this induces conformational changes

caused by slight rearrangements of the subunits. This could lead to alterations in the surface charge and may also expose new antigenic sites. It has been suggested that iron-rich ferritin molecules are taken up by lysosomes and a change in conformation may be the signal for this process to occur.

GENERAL DISCUSSION

GENERAL DISCUSSION

For many years now there has been a long-standing controversy over the number of types of subunit present in the apoferritin molecule.

Evidence based on electrophoretic techniques has lead Drysdale and his colleagues to propose a two subunit model comprising of an L subunit with a molecular weight of 19,000 and and H subunit with a molecular weight of 21,000, with varying proportions of these two subunits forming a 24 subunit apoferritin molecule. Drysdale has suggested that these two subunits have different properties, thus combining them in differing proportions gives rise to apoferritin molecules with different properties, namely isoferritins. Immunological data has provided further evidence for two types of subunit as it has been shown that ferritins from numerous tissues of the same species have different properties based on their reactivity with antibodies raised against a single tissue.

Drysdale's model is, in the main, based on techniques which utilize the surface properties of proteins for example isoelectrofocusing and immunoprecipitation. The surface charge and properties of any protein can be greatly influenced by factors other than its underlying

polypeptide sequence. Most post-translational modification events will affect the position to which a protein will move on isoelectrofocusing. Cragg and co-workers have shown that the extent of glycosylation of human serum ferritins influences the number of isoferritins seen on isoelectrofocusing. Therefore results from techniques of this type must be interpreted with caution as it could be possible that isoferritins have arisen solely as the result of post-translational events.

However, Drysdale has shown that two different molecular weight subunits can be seen on SDS gradient-pore polyacrylamide gels. This does not necessarily mean that the two subunits exist in the final assembled molecule. It may be that one subunit is a precursor of the other which is sufficiently stable to be isolated during purification procedures.

It is difficult to envisage how in a compact 24 subunit molecule additional protein material up to 24 x 2000 in molecular weight can be accomodated if subunit-subunit interactions, channels for the entry and release of iron and catalytic sites are all to be preserved. Additional material could be present on the surface of the molecule or in the central cavity, but in the latter case this would take up valuable storage space for iron.

In recent years there has been an increasing amount of evidence that the H and L subunits seen on SDS polyacrylamide gels are the products of two different genes. Peptide mapping experiments performed on purified H and L subunits show that these subunits have similar but not identical peptide compositions (Arosio, Adelman and Drysdale, 1978). This indicates that extensive homologies occur between the sequences of the two subunits but again this technique is influenced by the possibility of glycosylation and proteolytic processing. More recently the amino acid composition of H and L subunits have been determined by Otsuka, Maruyama and Listowsky (1981), their results show that the two subunits are similar but the larger H subunit is reported to have fewer leucine, phenylalanine and arginine residues. It therefore seems improbable that H subunits are the precursors of L subunits.

Otsuka, Maruyama and Listosky (1981) have also shown that purified H subunits from human liver can re-assemble in a well-defined manner to form particles which resemble natural apoferritin in electron micrographs. Circular dichroism data has indicated that homopolymers of H subunits have substantially less ordered secondary structure and a lower α helical content than their L counterparts.

If the H and L subunits are products of different genes with different polypeptide sequences then the varying proportions of these two subunits within the apoferritin molecule will obviously influence any chemical modification studies. The chemical modification and cleavage procedures carried out in this study have been performed on horse spleen apoferritin which is predominantly L subunits or on preparations of purified L subunits. The results obtained in these studies of one tryptophan, two cysteine and 5-6 tyrosine residues are in agreement with the published sequence of horse spleen apoferritin, i.e. the L subunit.

Chemical modification studies on apoferritins from other tissues such as liver, kidney and heart, which have a higher proportion of H subunits need to be carried out on purified subunits in order to obtain meaningful data. In order to obtain pure populations of either subunit a dissociation step is required. Although this does not necessarily effect the results of chemical modification and cleavage experiments, especially if they are performed in the presence of a denaturant, it does affect topographical studies. Studies on the topography of apoferritin would need to be carried out on native molecules and the results of these experiments would be difficult to interpret. Even if re-assembled H or L homopolymers were used, the dissociation and re-association procedure may affect the conformation of the subunits so they are no longer identical to those of the native molecule.

One way of resolving the long-standing dispute over the existence, or non-existence of two distinct polypeptides in apoferritin would be to look at the sequences of the H and L subunits seen on SDS polyacrylamide gels. Although initial studies carried out here were inconclusive and lack of time prevented further investigation, they warrent further study. If these two bands do have different sequences then they provide a molecular explanation for the microheterogeneity seen on isoelectrofocusing. This data would also provide information on the conserved sequences of the polypeptide chain which would suggest that these amino acids are involved in important regions of the subunit i.e. subunit-subunit interactions, catalytic and iron binding sites etc. Wustefeld and Crichton (1982) have recently published the sequence of human spleen apoferritin. When compared to the sequence of horse spleen apoferritin only 25 of the 174 amino acids in the sequence are changed, indicating that it is highly conserved from one species to another. They have also sequenced minor components of human spleen which are homologous, but not identical, to the major component. It therefore seems likely that the minor component is the 'H' subunit.

Another approach has been taken by Watanabe and Drysdale (1981) who have isolated poly A enriched RNA from HeLa cells (H-rich) and rat liver (L-rich). When

translated in wheat germ lysates they found that ferritin from HeLa RNA was predominantly H while that from liver RNA was predominantly L. These results indicate that H and L subunits in human and rat ferritins are derived from distinct mRNA species rather than post-translational modification.

Caskey *et al* (1983) have been able to assign the human ferritin gene to chromosome 19. If in the future further studies are carried out on DNA sequencing, protein sequencing and the translation of mRNA's then the controversy over H and L subunits will finally be resolved.

If two subunits do exist then it is likely that they have slightly different functions. It has been suggested that the 'H' subunits are preferentially formed under conditions of high iron dosage. The tissue culture experiments carried out here set out to investigate if Morris hepatoma cells synthesized ferritin in response to iron and to determine if one subunit was preferentially synthesized. The results clearly show that ferritin is synthesized as a response to the incorporation of iron into the culture media. Although no data was obtained supporting the preferential synthesis of either subunit, further studies of this type would be very valuable in providing information on the function of the subunits. If results from experiments of this type could then be correlated to DNA, and protein sequence analysis, then this would provide

not only a molecular basis for the structure and function of apoferritin but also a detailed explanation how the structure and function of the molecule changes in response to the metabolic demands of the individual.

SUMMARY

Summary

The majority of iron stored in the body is found in the form of ferritin. This iron storage protein enables the body to have an accessible store of iron for its metabolic functions and yet protect the body from the toxic effects of free iron. The molecule consists of 24 subunits which form a hollow shell into which iron is deposited or released depending on the metabolic state of the individual.

Horse spleen apoferritin was primarily used in this study. It is one of the most extensively investigated of all the ferritins as the electron density map at 2.8Å and the primary sequence are now well established.

The complex electrophoretic band pattern which is evident when horse spleen apoferritin is electrophoresed on gradient pore SDS polyacrylamide gels was investigated. Several types of polyacrylamide gradients, both step-wise and continuous, were initially investigated to determine which system gave the best resolution. A 6-22% polyacrylamide gradient was thought to give the best separation of protein and peptide bands. Using this system horse spleen apoferritin resolves into several protein bands with molecular weights above and below (15,000, 11,000 and 7,000) that of the subunit. By incorporating thiol into the sample buffer the presence and absence of certain bands could be influenced. This was interpreted as

being due to the presence of inter- and intra-molecular disulphide bonds. This was confirmed by devising a 2-dimensional diagonal technique using horizontal slab gel electrophoresis in the presence of SDS. Using a 15% T, 5% C SDS-polyacrylamide gel a sample of horse spleen apoferritin (prepared without thiol) was applied to one corner of the gel and electrophoresed. The gel was then overlayed with 10% (v/v) 2-mercaptoethanol and, following a short incubation, electrophoresed at right angles to the first dimension. The result of this experiment showed that

i) subunit dimers containing an intermolecular disulphide bond can exist as witnessed by the dimer into subunit conversion and

ii) the 15,000 molecular weight species represents subunit with an intact intramolecular disulphide bond as witnessed by conversion from 15,000 molecular weight state to the 19,000 molecular weight species on treatment with 2-mercaptoethanol.

The smaller molecular weight species have previously been reported to be 11,000 and 8,000 using uniform porosity SDS polyacrylamide gel electrophoresis. Using a gradient-pore system the molecular weights of these peptides were re-assessed and found to be 11,000 (peptide B) and 4,500 (peptide C). Amino acid analysis of peptide C indicated that this peptide had arisen from the C-terminal end of the subunit. On sequencing of the first six amino acids this was found to correspond well

with the last six amino acids of the published sequence. This would suggest that this peptide extends beyond the published sequence. One possible explanation would be that it is part of a longer sequence, perhaps derived from a larger subunit, for example the H subunit proposed by Drysdale. Further work would need to be carried out to establish this.

The possibility of subunit glycosylation influencing the electrophoresis of horse spleen apoferritin was investigated using an affinity column for glycoproteins. Three preparations were isolated using this approach.

1) non-glycoslated

2) non-glycosylated but retained to support via disulphide bonds to glycosylated subunits

3) glycosylated

When these three preparations were run on SDS gradient-pore polyacrylamide gels no appreciable differences could be observed. However glycosylation of subunits did occur but not to an extent which would affect the molecular weight of the subunit. This may, however, influence other electrophoretic techniques such as isoelectrofocusing and also immunological studies.

Several chemical modification studies were carried out to determine the number, and also possible location, of certain amino acids within the subunit molecule.

Using the reagent tetranitromethane the nitration of tyrosine residues over a range of temperatures was investigated. Below 37 °C only one tyrosyl residue was nitrated however on increasing the temperature up to 60 °C the number of nitrotyrosyl residues rose to 5.75. The possible location of the single tyrosine residue which was nitrated below 37 °C was determined by examining electron density maps of horse spleen apoferritin in conjunction with the primary sequence. Using this approach tyrosine at position 8 appeared to be the most likely candidate as it is in a non-helical region at the N-terminus. The remaining tyrosine residues appear to be in regions of α helices which are inaccessible to tetranitromethane at low temperatures but are nitrated with increasing temperatures. This observation is probably a result of the increased kinetic energy of the reactants together with a conformational change of the subunit at elevated temperatures.

The number of tryptophan residues has been reported to be either one or two residues per subunit. It was decided to re-assess the number of tryptophan residues by cleavage of the subunit with o-iodosobenzoic acid, a reagent which cleaves at the carboxyl side of tryptophan. When the chemically digested material was applied to SDS polyacrylamide gel electrophoresis only one band was present with a molecular weight of 9-10,000. On examination of the sequence data of Heusterpreute and

Crichton a tryptophan residue is thought to occur at position 89. Cleavage at this position would result in two peptides of approximately equal size and hence would be expected to give one band on SDS-polyacrylamide gel electrophoresis. However this does not rule out the possibility of two adjacent tryptophan residues being present in the molecule.

To complement the chemical modification and cleavage studies carried out on tyrosine and tryptophan residues a second derivative spectrophotometric method was also used. Using this technique the molar ratio of tyrosine to tryptophan can be determined, and in the case of horse spleen apoferritin was found to be 1.42 ± 0.15 tryptophan residues and 5.57 ± 0.12 tyrosine residues per subunit. These spectrophotometric results provide good evidence to support the view that there are 6 tyrosine residues and one tryptophan residue per subunit, values which are in agreement with the sequence data.

The effect of thiol on the SDS electrophoretic profile of horse spleen apoferritin led to a determination of the number of cysteine residues using 2-nitro-5-thiocyano-benzoic acid, in order to understand how disulphide bond formation may occur. 2-nitro-5-thiocyanobenzoic acid cleaves at the C-terminal side of cysteine residues. Using this reagent only two peptides were found to occur on SDS-polyacrylamide gel. This would be consistent with

only one cysteine residue. However, addition of the molecular weights of these bands does not correspond to the molecular weight of the subunit. One explanation of this therefore is that the smaller molecular weight band contained two peptides of similar molecular weight and that there are two cysteine residues in a subunit of horse spleen apoferritin. This agrees well with the number and also location of cysteine residues in the published sequence.

Many tissue ferritins contain multiple forms of ferritin, termed isoferritins, which appear to differ structurally and immunologically. The isoferritins are thought to represent varying proportions of two subunits 'H' and 'L' forming a 24-mer, however the existence of two subunits is in dispute. Using preparative SDS gradient-pore polyacrylamide gel electrophoresis samples were obtained which contained 'L' subunits and 'H'-rich subunits. When attached to a solid phase support and sequenced on a LKB 4030 solid phase sequencer the sample of L subunits was resistent to sequencing. This was expected as it is known that the N-terminal amino acid is acetylated. When the sample of H-rich subunits was sequenced this was also resistent to sequencing. This would indicate that the H subunits are also blocked at the N-terminus. Unfortunately due to insufficient time it was not possible to remove the blocking group from the N-terminus and sequence inwards.

An approach of this type may throw light on the existence/non-existence of H and L subunits.

Tissue culture experiments using Morris hepatoma cells clearly showed that addition of iron to the growth medium increased the ferritin level of these cells up to five times that of the control. When samples of iron-induced ferritin were run on SDS-polyacrylamide gels the existence of two subunits could be demonstrated. The incorporation of ^{14}C-leucine into cells incubated with different concentrations of iron supplemented media was also studied. Labelled ferritin was precipitated immuno-chemically, then subjected to SDS electrophoresis and fluorimetry. In the initial experiments insufficient label was added to allow any interpretation of the results to be valid. These results were obtained while on a 2-month visit to Prof. Listowsky's laboratory at Albert Einstein College of Medicine, New York. Unfortunately, owing to limited facilities at Dundee, it was not found possible to continue with this line of enquiry. However using an approach of this type may lead to an explanation of the different functional roles of the two subunits.

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